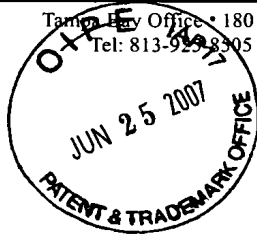


06-26-07
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June 25, 2007

Mail Stop Appeal Brief – Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Applicant: John H. Paul

Serial No.: 10/707,747

Filing Date: 01/08/2004

For: Detection of Red Tide Organisms by Nucleic Acid Amplification

Our Reference: 1372.120.PRC

Examiner: David C. Thomas

Art Unit: 1651

Confirmation No.: 1746

Dear Sir:

Enclosed please find the following:

1. Brief of Appellant having a Certificate of Mailing dated June 25, 2007;
2. Exhibit A – A Single Origin of the Peridinin- and Fucoxanthin- Containing Plastids in Dinoflagellates through Tertiary Endosymbiosis;
3. Exhibit B – A Single Origin of the Peridinin- and Fucoxanthin- Containing Plastids in Dinoflagellates through Tertiary Endosymbiosis – Support Information Table 1;
4. Exhibit C - A Single Origin of the Peridinin- and Fucoxanthin- Containing Plastids in Dinoflagellates through Tertiary Endosymbiosis – Support Information Table 2;
5. Exhibit D – GenBank Accession No. AY119786;
6. Exhibit E – Center for Culture of Marine Phytoplankton Catalog, CCMP718 Karenia brevis;
7. Exhibit F – Design Strategies and Performance of Custom DNA Sequence Primers;
8. Exhibit G – In re Deuel;
9. Credit Card Payment form PTO-2038 in the amount of \$250.00; and.
10. Self-addressed, postage prepaid post card to serve as a receipt for items 1-9.

Very respectfully,

SMITH & HOPEN

By: Thomas E. Toner

tom.toner@smithhopen.com

TET/at
Enclosures

CERTIFICATE OF MAILING
(37 C.F.R. 1.10)

I HEREBY CERTIFY that this Appeal Brief is being deposited with the United States Postal Service as "Express Mail Post Office to Addressee" Mailing Label No. EM057595284US, addressed to: Mail Stop Appeal Brief - Patents, Commissioner for Patents, P.O. Box 1450 Alexandria, VA 22313-1450 on June 25, 2007

Date: June 25, 2007

April Turley
April Turley



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Serial No. : **10/707,747** Confirmation No. **1746**
Applicant : **John H. Paul**
Filed: : **01/08/2004**
TC/A.U. : **1651**
Examiner : **David C. Thomas**
Docket No. : **1372.120.PRC**
Customer No. : **21,901**
For : **Detection of Red Tide Organisms by Nucleic Acid Amplification**

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313

FIRST AMENDED BRIEF OF APPELANT

Sir/Madam:

In furtherance of its appeal from the Non-Final Rejection mailed 23 January 2007,
Applicant hereby submits its Appeal Brief.

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1. REAL PARTY IN INTEREST

The real party in interest is the University of South Florida, the assignee of record, which is a state university, organized under the laws of the State of Florida, as evidenced by the assignment set forth at Reel 013396, Frame 0478.

2. RELATED APPEALS AND INTERFERENCES

None

3. STATUS OF CLAIMS

Rejected claims:	16-21, 24-30
Canceled claims:	1-15, 22-23
Withdrawn Claims:	31-36
Claims under appeal:	16-21, 24-30

4. STATUS OF AMENDMENTS

No amendments have been made subsequent to the non-final rejection by the Office dated January 23, 2007.

5. SUMMARY OF CLAIMED SUBJECT MATTER

Citations to the specification are by page and line number. A concise explanation of the invention defined in the claims involved in this appeal is provided below. Claim 16 is the only independent claim on appeal.

Claim 16 recites a method for screening a sample for the presence of *K. brevis* (page 1 , lines 28-29), comprising: subjecting the sample to amplification using a pair of oligonucleotide primers (page 7, lines 14-16) capable of amplifying a target region of the ribulose 1, 5-biphosphate carboxylase large subunit (rbcL) of *K. brevis* (page 1, lines 29-31); and assaying the mRNA for the presence of the amplified target region of the ribulose 1, 5-biphosphate carboxylase-oxygenase large subunit (rbcL) unique to *K. brevis* (page 2, lines 1-2).

6. GROUND OF REJECTION TO BE REVIEWED ON APPEAL

- I. Whether the Office erred in rejecting claims 16 – 18 under 35 U.S.C. §103(a) as being rendered obvious by Yoon et al. in view of Buck et al. and further in view of GenBank Accession No. AY119786.¹
- II. Whether the Office erred in rejecting claims 19-21, 24 and 25 under 35 U.S.C. §103(a) as being rendered obvious by Yoon et al. in view of Bowers et al. and further in view of Wilson et al. and further in view of Buck et al. and in still further view of GenBank Accession No. AY119786.²
- III. Whether the Office erred in rejecting claims 26-30 under 35 U.S.C. §103(a) as being rendered obvious by Yoon et al. in view of Leone et al. and further in view of Wilson et al. and further in view of Buck et al. and in still further view of GenBank Accession No. AY119786.³

¹ Non-Final Action, page 3

² Non-Final Action, page 6

³ Non-Final Action, page 11

7. ARGUMENT

- I. The Office erred in rejecting claims 16 – 18 under 35 U.S.C. §103(a) as being obvious over Yoon et al. in view of Buck et al. and further in view of GenBank Accession No. AY119786 because Yoon does not teach a method of detecting *K. brevis* in a sample.**

The Office has mischaracterized the teachings of Yoon. The Office alleges that Yoon teaches a method for screening a sample for the presence of *K. brevis*. (Office Action dated January 23, 2007, page 3, lines 7-8). Yoon, however, teaches a method of determining the origin of plastids in red algae and dinoflagellates. The *K. brevis* samples in Yoon were pure samples, therefore making the detection of *K. brevis* in a sample unnecessary. Table 1 of the supporting information shows that the *K. brevis* used was obtained from the Center for Culture of Marine Phytoplankton (CCMP 718). (see Table 1, Yoon et al., supporting information; attached hereto as Exhibit B). This culture, CCMP 718, was isolated in the 1960's. (CCMP catalog, CCMP718; attached hereto as Exhibit E). Therefore, the assertion that Yoon teaches a method for screening a sample for the *K. brevis* is at odds with the fact that Yoon used a pure *K. brevis* sample. Accordingly, any primer sequence derived from the *rbcL* gene, or any other gene for that matter, would amplify the pure culture of *K. brevis*. Yoon, at best, teaches a method of amplifying a pure sample rather than detecting the presence of *K. brevis* in an impure sample.

Next, the Office alleges the method disclosed by Yoon further comprises: using a pair of oligonucleotide primers capable of amplifying a target region of the ribulose 1,5-biphosphate carboxylase-oxygenase large subunit (*rbcL*) of *K. brevis* (amplification using species specific primers, page 11725, column 1, lines 13-24 and Table 2, supporting information). (Office Action dated January 23, 2007, page 3, line 19 through page 4, line 4). Yoon, in the relevant passage, states:

PCRs were done by using specific primers for each of the plastid genes (Table 2, which is published as supporting information on the PNAS web site). The presence of highly variable third codon positions in the *psaA* gene led us to use species-specific primers based on sequences in sister species. Because introns were found in the *psaA* gene of some red algae, the reverse transcription (RT)-PCR method was used to isolate cDNA. For the RT-PCR,

total RNA was extracted by using the RNeasy Mini kit (Qiagen). To synthesize cDNA from total RNA, Moloney murine leukemia virus Reverse Transcriptase (GIBCO/BRL) was used following the manufacturer's protocol. (Yoon, page 11725, column 1, lines 13-23, emphasis added).

Yoon does not teach the use of species specific primers for the detection of a dinoflagellate in a sample. Rather, Yoon used primers specific to the plastids being tested (namely, the *psaA*, *psaB* and *rbcL* genes) and not a particular species. Yoon only used species specific primers for detection of the *psaA* gene and not the *rbcL* gene. Table 2, on which the Office relies to support the finding that Yoon teaches species specific primers for use with the *rbcL* gene, indicates that all primer sequences used to amplify the *rbcL* gene are general with regard to specificity (with the exception of R-753 which is only specific to the family Bangiaceae). Yoon teaches only the use of non-specific (general) primers for use with the *rbcL* gene. Nothing in Yoon teaches that the *rbcL* gene of *K. brevis* contains a sequence that is unique to that species.

Next, the Office alleges that Yoon teaches "assaying the mRNA for the presence of the amplified target region of the [*rbcL*] unique to *K. brevis*." (Office Action dated January 23, 2007, page 3, lines 13-14). The Office continues:

PCR products generated from total RNA were sequenced using dye terminators as probes, p. 11725, column 1, lines 20-29; sequences are unique to *K. brevis*, p. 11726, column 1, lines 8-14 and Figure 1A and B and GenBank Accession No. AY119786; BLAST search indicates primers amplify a region unique to *K. brevis*, see BLAST results. (Office Action dated January 23, 2007, page 3, lines 14-18).

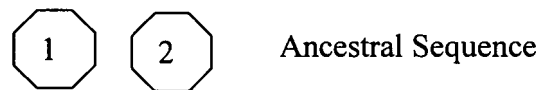
The passage upon which the Office relies to support the allegation that the "sequences were unique to *K. brevis*" is reproduced below:

These data provide strong support, therefore, for the monophyly of fucoxanthin-containing and haptophyte plastids. Our results, using an expanded data set of *rbcL* sequences, is consistent with previous reports (32). The two genera of fucoxanthin-containing dinoflagellates, *Karenia* and *Karlodinium*, are paraphyletic at the base of haptophyte clade, a result that is also found in the *psbA* tree. (Yoon, page 11726, col. 1, lines 8-14).

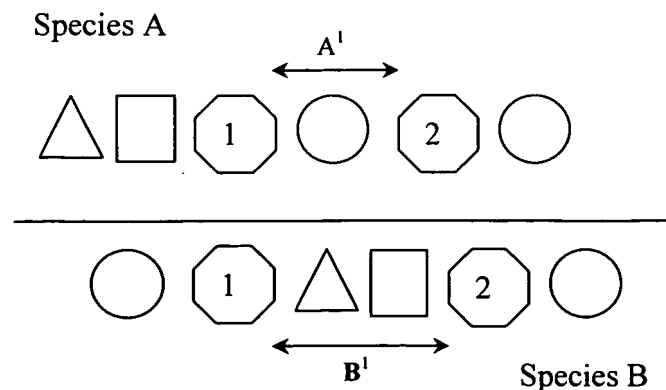
This passage is devoid of any statement that could lead one to conclude that the sequences used were unique to *K. brevis*. To the contrary, Yoon states it used “an expanded data set of *rbcL* sequences.” (Yoon, page 11726, column 1, line 10). The only conclusion to be drawn from this statement is that the genus *Karenia* (which includes both *K. brevis* and *K. mikimotoi*) and the genus *Karlodinium* contain some, but not all, of the descendants from a common ancestor (*i.e.*, they are paraphyletic). Rather than speak to the uniqueness of the *rbcL* gene of *K. brevis*, this passage shows the close relationship among the genus *Karenia* with the sister genus *Karlodinium* as well as others with regard to the *rbcL* gene.

The Office also relies on Figures 1A and 1B. (Yoon, page 11726). Figures 1A and B show a phylogeny of red algal and red algal-derived plastids using combined *psaA* and *psbA* sequences, the *rbcL* gene was not used. *Id.* Both Figures 1A and 1B were created using LogDet values (a.k.a., paralinear distances). Distance methods, such as LogDet, attempt to measure the number of changes per site since two species (sequences) split. LogDet analysis provides only distances between sequences but does not reveal sequences, unique or otherwise. The figures cited only show that the disclosed genera have varying distances between common sequences of the *psaA* and *psaB* plastids. It cannot be concluded from Yoon whether the sequences establishing the paralinear distance in each genera are unique to a species.

Consider the following example wherein sequence 1 and 2 are proximate in a given ancestor.



Varying sequences develop adjacent, and between, the formerly proximate sequence as the ancestor gives rise to diverging species.



As shown in this example, the evolution of different sequences between ancestral sequence 1 and 2 yields a variance in paralogous distances (A^1 and B^1) sufficient to distinguish Species A from Species B based on LogDet values. The LogDet values, however, represent only the distances between the ancestral sequences and not the sequences themselves. Moreover, although the sequences occur in different loci, no sequence shown in either species is unique.

Figure 2B, which was created using LogDet values of the *rbcL* gene, shows that several species have identical LogDet values on the species level (species identities were known prior to testing). (Yoon, page 11727). As shown, *K. brevis*, *K. mikimotoi*, *Pavlova lutherii*, and *Pavlova gyraus* all have LogDet values of 100 on the species level. Therefore, the method employed by Yoon is not capable of detecting *K. brevis* in a sample because numerous species would provide false positives using the methods of Yoon.

Next, the Office claims that a BLAST search performed by the Office indicates that the primers taught by Yoon amplify a region unique to *K. brevis*. (Office Action dated January 23, 2007, page 3, lines 17-18). The Office alleges that “upon further analysis of the sequence amplified by the *rbcL* gene primers taught by Yoon (Table 2), the amplified target region sequence is unique to *K. brevis* based on a BLAST search of the 158-basepair amplicon (see BLAST search results) and does not share extensive homology with *K. mikimotoi*.” (Office Action dated January 23, 2007, page 16, lines 8-12).

While the Applicant appreciates the efforts of the Office, the BLAST search is an exercise in hindsight. A BLAST search performed by an examiner during examination does not establish that a primer sequence disclosed in a reference was known to be unique at the time the reference was authored or at the time the invention was made. As previously established, Yoon described all primer sequences for the *rbcL* gene as general (see Table 2, Yoon et al. supporting information; attached hereto as Exhibit C). Even assuming the Office’s findings are correct, the Office has not established that it was known at the time the invention was made that the primers taught by Yoon would amplify a sequence of the *rbcL* gene unique to *K. brevis*. The BLAST search is also insufficient because it only reveals that *K. brevis* does not share extensive homology with *K. mikimotoi*, only one other species. The BLAST search does not establish the amplified region is unique across a range of species.

Lastly, the Office relies on GenBank Accession No. AY119786 which lists 907 base pairs, less than two-thirds of *Karenia*’s *rbcL* gene, of linear DNA derived from the *rbcL* gene of

K. brevis. (Application, page 3, line 28). Nothing contained within the NCBI database, however, indicates that any of the sequences listed are unique to *K. brevis* or that the *rbcl* gene is a likely candidate for containing a unique sequence. Yoon discloses that the Form I *rbcl* gene is the primitive (i.e., not derived) condition in dinoflagellates, emphasizing its ubiquitous nature. There is also no teaching of what portion, if any, of the sequence deposited in the GenBank Database is unique to *K. brevis*, or of any method to determine same. Simply stated, the references do not teach or infer that a unique sequence exists on the *rbcl* gene.

As Applicant has shown, Yoon does not teach a method of detecting the presence of *K. brevis* in a sample. Yoon does not teach a sequence of the *rbcl* gene that is unique to *K. brevis*, or that the *rbcl* gene contains a unique sequence. Assuming, *arguendo*, that the primer sequences taught by Yoon amplify a region of the *rbcl* gene that is unique to *K. brevis*, nothing in Yoon indicates such and, therefore, one of ordinary skill in the art at the time the invention was made would not be motivated to use the sequences taught by Yoon. Moreover, there was no reasonable expectation of success based on the fact that Yoon is silent as to the uniqueness of the *rbcl* gene of *K. brevis* as well as Yoon's categorization of the *rbcl* primers as general. The remaining references are moot regarding the claimed invention and do not support a finding of obviousness with or without Yoon.

For these reasons, Yoon et al. in view of Buck et al. and further in view of GenBank Accession No. AY119786 fail to render the claimed invention obvious. It is therefore respectfully requested that the rejection under 35 U.S.C. 103(a) be reversed.

II. The Office erred in rejecting claims 19-21, 24 and 25 under 35 U.S.C. §103(a) as being obvious over Yoon et al. in view of Bowers et al. and further in view of Wilson et al. and further in view of Buck et al. and in still further view of GenBank Accession No. AY119786 because Yoon does not teach any of the elements of claim 16.

The Office asserts that "it would have been *prima facie* obvious... to combine the methods of Yoon, Bowers and Wilson [because] Yoon teaches the detection of a sequence specific to *K. brevis* in the *rbcl* gene using a reverse-transcriptase polymerase chain reaction method," and "Bowers describes a real-time polymerase chain reaction using an internal fluorescent probe to detect harmful dinoflagellates in a rapid, homogenous assay," and lastly that "Wilson teaches methods to design species-specific primers in order to detect single species in samples containing

many different related or unrelated species.” (Office Action dated January 23, 2007, page 8, lines 10-17). As previously demonstrated, Yoon does not teach a method of detecting *K. brevis* at all.

The Office then concludes, without factual support, that “an ordinary practitioner would have been motivated to combine these methods to provide a rapid test for harmful algal bloom species that results in an added level of specificity compared with assays based on traditional PCR methodology.” (Office Action dated January 23, 2007, page 8, lines 17 -20).

The specific primers claimed are rejected by reliance on the “recent” court decision *In re Deuel*. (51 F.3d 1552 (Fed. Cir. 1995). In *Deuel*, as admitted by the Office, the Court of Appeals for the Federal Circuit determined that the “existence of a general method of identifying a specific DNA does not make the specific DNA obvious.” (Office Action dated February 28, 2006, page 16, lines 10-12). It is not clear, however, how this case supports the position that the mere existence of a general method of using sequence information for differentiating among taxa would make a primer for a species-specific sequence obvious.

The Office quotes from *Deuel*, stating that “a prior art compound may suggest its homologs because homologs often have similar properties and therefore chemists of ordinary skill would ordinarily contemplate making them to try to obtain compounds with improved properties.” *Id.* at 17-20. The following paragraph from *Deuel*, which was not provided by the Office, however, states:

In all of these cases, however, the prior art teaches a specific, structurally-definable compound and the question becomes whether the prior art would have suggested making the specific molecular modifications necessary to achieve the claimed invention. (*In re Deuel* at 1558).

The references, alone or in combination, do not disclose a specific, structurally-definable sequence unique to *K. brevis*.

Moreover, *Deuel* unambiguously states that “a *prima facie* case of unpatentability requires that the teachings of the prior art suggest *the claimed compounds* to a person of ordinary skill in the art” when new chemical entities are claimed in structural terms. (*Id.* at 1557, emphasis in original).

Deuel makes it clear that “while the general idea of the claimed molecules, their function, and their general chemical nature may have been obvious from the prior art, and that the knowledge that some sequence exists, the precise molecules are not obvious without a teaching of the claimed, or closely related, molecule. (*Id.* at 1558). The Court expressly stated that “[a] general motivation to search for some gene that exists does not necessarily make obvious a

specifically-defined gene that is subsequently obtained as a result of that search.” (*Id.*). Here, as was the case in *Deuel*, more is needed to support a finding of obviousness. (*Id.*).

The Office next asserts that Buck provides evidence of the equivalence of primers. (Office Action dated January 23, 2007, page 10, line 8; page 15, line 2). Applicant respectfully points out that none of the references teach a specific primer of which the claimed invention can be an equivalent. The Office continues, however, placing the following “emphasis” on Buck’s findings:

Buck found that EVERY SINGLE PRIMER worked (see page 533, column 1). Only one primer ever failed, No. 8, and that primer functioned when repeated. Further, EVERY SINGLE CONTROL PRIMER functioned as well (see page 533, column 1). (Office Action dated January 23, 2007, page 10, lines 14 -17; page 15, lines 8-11, emphasis in original).

Applicant respectfully disagrees with the Offices characterization of Buck. The broad interpretation given to Buck by the Office would render all primers obvious. The rejection also disregards the unobvious and indispensable element of deciding which sequence to amplify. Just as it is improper to pick and choose elements from the prior art to construct a finding of obviousness, it is improper to pick and choose portions of a particular reference without considering the reference as a whole. An accurate characterization of Buck’s findings is clearly outlined in the abstract.

We conclude that under *optimal sequencing conditions* with *highly pure template and primer*, many of the commonly applied primer design parameters are dispensable.” (Buck, page 528, Abstract, emphasis added).

In Buck, a panel of 95 primers were synthesized as controls and 69 primers were submitted as tests. (Buck, page 530, column 1). “The plasmid template was *preselected* to contain a test sequence *lacking obstacles to sequence extension...* and *purified* by double banding in CsCl-ethidium bromide isopycnic density gradients.” (Buck, page 530, column 2, emphasis added). In short, the template was cherry-picked for success, as evidenced by the statement in Buck that “this template was extremely pure and optimal for sequencing.” (*Id.* at 535, column 2). Creating an ideal environment with a template consisting of best-case scenario design can hardly be equated to a real-world environment. Buck clearly states that template purity and technical expertise “possibly played greater rolls” in the success experienced, rather than the obviousness of the primers. (*Id.* at 535, column 2). The Office has also disregarded the disclaimer:

Different results may be obtained using less carefully purified DNA templates with unusual sequences or structures in less rigorously controlled sequencing operations. (*Id.* at 536, column 1).

Regardless of the inadequacies of the *prima facie* case, claims 19-21, 24 and 25 depend from claim 16. Claim 16 has been shown to be nonobvious over the prior art, *supra*. It is well settled that if an independent claim is nonobvious under 35 U.S.C. §103(a), then any claim depending therefrom is nonobvious as a matter of law. (MPEP §2143.03; see also *In re Fine*, 837 F.2d 1071 (Fed. Cir. 1988)). Therefore, the rejection of claims 19-21, 24 and 25 is improper and should be reversed.

IV. The Office erred in rejecting claims 26-30 under 35 U.S.C. §103(a) as being obvious over Yoon et al. in view of Leone et al. and further in view of Wilson et al. and further in view of Buck et al. and in still further view of GenBank Accession No. AY119786 because Yoon does not teach any of the elements of claim 16.

Lastly, claims 26-30 stand rejected under 35 U.S.C. §103(a) as being anticipated by Yoon et al. in view of Leone et al. and further in view of Wilson et al. and further in view of Buck et al. and in still further view of GenBank Accession No. AY119786. Here the Office asserts that Yoon, in view of Buck and GenBank Accession No. AY119786, teaches the limitations of claims 16-18. Applicant has shown, *supra*, this is not the case.

The Office then alleges that, with regard to claims 27 and 30, Yoon teaches that the primers specific to a target region of the *K. brevis* *rbcL* gene consisting of SEQ. ID. No. 4 (Table 1 and GenBank Accession No. AY119786, positions 733-751) and SEQ. ID. NO. 5 (Table 1 and GenBank Accession No. AY119786, positions 819-798) to generate an 87-base pair amplicon (from positions 733 to 819 of GenBank Accession No. AY119786). (Office Action dated January 23, 2007, page 11, lines 10-18). Here, the Office claims the primer sequences, SEQ. ID. NOs. 4 and 5, are disclosed in the DNA disclosed in GenBank Accession No. AY119786, positions 733-751 and positions 819-798. *Id.* The Office points to the same DNA sequence to show both the primers and the target, which is scientifically flawed. Moreover, Applicant does not claim the DNA sequences of the *rbcL* gene but rather a primer having a sequence *consisting* of SEQ. ID. NO. 4 and SEQ. ID. NO. 5. The Office has not shown why an artisan would select primers which *consist* of those sequences.

Regardless of the inadequacies of the *prima facie* case, claims 26-30 depend from claim 16. Claim 16 has been shown above to be nonobvious over the prior art. It is well settled that

if an independent claim is nonobvious under 35 U.S.C. §103(a), then any claim depending therefrom is nonobvious as a matter of law. (MPEP §2143.03; see also *In re Fine*, 837 F.2d 1071). Therefore, the rejection of claims 26-30 is improper and should be reversed.

8. CLAIMS APPENDIX

Serial No: 10/707,747
Filed: 08 January 2004
Title: Detection of Red Tide Organisms by Nucleic Acid Amplification

REJECTED CLAIMS

16. A method for screening a sample for the presence of *K. brevis*, comprising:
subjecting the sample to amplification using a pair of oligonucleotide primers capable of amplifying a target region of the ribulose 1, 5-biphosphate carboxylase-oxygenase large subunit (rbcL) of *K. brevis*; and
assaying the mRNA for the presence of the amplified target region of the ribulose 1, 5-biphosphate carboxylase-oxygenase large subunit (rbcL) unique to *K. brevis*.
17. The method of claim 16 wherein the pair of oligonucleotide primers specifically amplify mRNA of a target region of the ribulose 1, 5-biphosphate carboxylase-oxygenase large subunit (rbcL) of *K. brevis* and do not amplify a region of the ribulose 1, 5-biphosphate carboxylase-oxygenase large subunit (rbcL) of *K. mikimotoi*.
18. The method of claim 16 wherein the target region of the ribulose 1, 5-biphosphate carboxylase-oxygenase large subunit (rbcL) of *K. brevis* is about 87 to 91 base pairs in length
19. The method of claim 16 wherein the amplification process is selected from the group consisting of real-time reverse-transcriptase polymerase chain reaction and quantitative thermocycling.
20. The method of claim 19 wherein the pair of oligonucleotide primers consist of SEQ. ID. No. 1 and SEQ. ID. No. 2.
21. The method of claim 20 wherein the pair of oligonucleotide primers are specific to a target region of the ribulose 1, 5-biphosphate carboxylase-oxygenase large subunit (rbcL) of *K. brevis* about 91 base pairs in length.
24. The method of claim 20 wherein the amplification process is applied to the sample in the presence of a probe.
25. The method of claim 24 wherein the probe consists of SEQ. ID. No. 6.

26. The method of claim 16 wherein the amplification process is real time nucleic acid sequence based amplification.
27. The method of claim 26 wherein the pair of oligonucleotide primers consist of SEQ. ID. No. 4 and SEQ. ID. No. 5.
28. The method of claim 26 wherein the amplification process is applied to sample in the presence of a probe.
29. The method of claim 28 wherein the probe comprises a nucleotide sequence consisting of SEQ. ID. No. 3.
30. The method of claim 26 wherein the pair of oligonucleotide primers is specific to a target region of the ribulose 1, 5-biphosphate carboxylase-oxygenase large subunit (rbcL) of *K. brevis* about 87 base pairs in length.

9. EVIDENCE APPENDIX

- Exhibit A: Yoon et al., *A Single Origin of the Peridinin- and Fucoxanthin-Containing Plastids in Dinoflagellates Through Tertiary Endosymbiosis*; (Proc. Natl. Acad. Sci. USA. (2002) 99: 11724-11729).
- Exhibit B: Yoon et al., *A Single Origin of the Peridinin- and Fucoxanthin-Containing Plastids in Dinoflagellates Through Tertiary Endosymbiosis*, Supporting information, Table 1;
<http://www.pnas.org/cgi/content/full/172234799/DC1/1>.
- Exhibit C: Yoon et al., *A Single Origin of the Peridinin- and Fucoxanthin-Containing Plastids in Dinoflagellates Through Tertiary Endosymbiosis*, Supporting information, Table 2;
<http://www.pnas.org/cgi/content/full/172234799/DC1/2>
- Exhibit D: GenBank Accession No. AY119786;
<http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&id=21913674>
- Exhibit E: Center for Culture of Marine Phytoplankton catalog, CCMP718 *Karenia brevis*;
<http://ccmp.bigelow.org/SD/display.php?strain=CCMP718&genus=Karenia&Species=brevis&Class=Dinophyceae>
- Exhibit F: Buck et al., *Design Strategies and Performance of Custom DNA Sequencing Primers*; (Biotechniques (1999) 27: 528-536).
- Exhibit G: *In re Deuel*, 51 F.3d 1552, 63 USLW 2624, 34 U.S.P.Q.2d 1210 (C.A.F.C., 1995).

Exhibit A

A single origin of the peridinin- and fucoxanthin-containing plastids in dinoflagellates through tertiary endosymbiosis

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The most widely distributed dinoflagellate plastid contains chlorophyll c_2 and peridinin as the major carotenoid. A second plastid type, found in taxa such as *Karlodinium micrum* and *Karenia* spp., contains chlorophylls $c_1 + c_2$ and 19'-hexanoyloxy-fucoxanthin and/or 19'-butanoyloxy-fucoxanthin but lacks peridinin. Because the presence of chlorophylls $c_1 + c_2$ and fucoxanthin is typical of haptophyte algae, the second plastid type is believed to have originated from a haptophyte tertiary endosymbiosis in an ancestral peridinin-containing dinoflagellate. This hypothesis has, however, never been thoroughly tested in plastid trees that contain genes from both peridinin- and fucoxanthin-containing dinoflagellates. To address this issue, we sequenced the plastid-encoded *psaA* (photosystem I P700 chlorophyll *a* apoprotein A1), *psbA* (photosystem II reaction center protein D1), and "Form I" *rbcl* (ribulose-1,5-bisphosphate carboxylase/oxygenase) genes from various red and dinoflagellate algae. The combined *psaA* + *psbA* tree shows significant support for the monophyly of peridinin- and fucoxanthin-containing dinoflagellates as sister to the haptophytes. The monophyly with haptophytes is robustly recovered in the *psbA* phylogeny in which we increased the sampling of dinoflagellates to 14 species. As expected from previous analyses, the fucoxanthin-containing dinoflagellates formed a well-supported sister group with haptophytes in the *rbcl* tree. Based on these analyses, we postulate that the plastid of peridinin- and fucoxanthin-containing dinoflagellates originated from a haptophyte tertiary endosymbiosis that occurred before the split of these lineages. Our findings imply that the presence of chlorophylls $c_1 + c_2$ and fucoxanthin, and the Form I *rbcl* gene are in fact the primitive (not derived, as widely believed) condition in dinoflagellates.

One of the most intriguing stories in plastid evolution is found in the dinoflagellate algae. This diverse, predominantly unicellular group is characterized by having one transverse and one longitudinal flagellum and a distinct layer that lies beneath the cell membrane (the amphiesma). Only about one-half of dinoflagellates are photosynthetic and many of these species are mixotrophic (1). Some heterotrophic dinoflagellates have acquired a temporary plastid in their cytoplasm (2). Others, such as *Symbiodinium* spp., are themselves endosymbionts of corals (3). Regardless of trophic condition, the dinoflagellates are an important component of marine ecosystems as symbionts and primary producers, and as the main source of toxic red tides (1, 4).

Photosynthetic dinoflagellates contain several types of plastids. The most common type is a 3-membrane bound plastid that contains chlorophyll c_2 with peridinin as the main carotenoid (5, 6). Secondary endosymbiosis, in which a photosynthetic eukaryote (in this case, a red alga) was engulfed by a nonphotosynthetic protist, is widely accepted as the origin of this plastid (7–11). Peridinin is believed to have evolved in the plastid of the ancestral dinoflagellate, with other plastid types being subsequent replacements of this organelle through tertiary (the uptake of an alga containing a secondary endosymbiont) endosymbiosis (11). A second type of plastid found in *Karenia brevis*

(as *Gymnodinium breve*), *Karenia mikimotoi* (as *Gymnodinium mikimotoi*), and *Karlodinium micrum* (as *Gymnodinium galathea-num*) (12) is surrounded by three membranes and contains chlorophylls $c_1 + c_2$ and 19'-hexanoyloxy-fucoxanthin and/or 19'-butanoyloxy-fucoxanthin, but lacks peridinin (6, 13, 14). These taxa are believed to be monophyletic, and their plastid is believed to have originated from a haptophyte alga through a tertiary endosymbiosis in their common ancestor (15). Haptophyte algae are primarily unicellular marine taxa that have external body scales composed of calcium carbonate known as coccoliths, two anterior flagella, and plastids surrounded by four membranes. Haptophyte plastids also contain chlorophylls $c_1 + c_2$ and fucoxanthin (6). Tertiary endosymbiosis explains the origin of the plastid in several other dinoflagellates: cryptomonad-like plastid (i.e., *Dinophysis acuminata*; ref. 16), diatom-like plastid (i.e., *Peridinium foliaceum*; refs. 17 and 18), and prasinophyte-like plastid (i.e., *Lepidodinium viride*; ref. 19). Like *Karlodinium* and *Karenia*, all dinoflagellates containing anomalous plastids are thought to trace their ancestry to a peridinin-containing common ancestor (10, 15). A single study using limited photosystem II reaction center protein D1 (*psbA*) data has suggested otherwise, i.e., that both fucoxanthin and peridinin dinoflagellates may share a single plastid ancestor, although no haptophytes were included in this analysis (20).

The idea that plastids with fucoxanthin are the result of a replacement of the secondary, peridinin-containing plastid in *Karenia* and *Karlodinium* has yet to be rigorously tested by using phylogenies that contain sequence data from both types of plastids. To address this gap in our knowledge, we sequenced 36 *psaA* (photosystem I P700 chlorophyll *a* apoprotein A1), 36 *psbA*, and 23 "Form I" *rbcl* (ribulose-1,5-bisphosphate carboxylase/oxygenase) plastid-encoded coding regions from various red and dinoflagellate algae. These sequences were analyzed to infer a phylogeny with both peridinin- and fucoxanthin-containing dinoflagellate plastids in a context of broad taxon sampling.

Materials and Methods

Algal Cultures and Sequencing. The algal cultures were obtained from the Culture Collection of Algae and Protozoa (CCAP; Dunbeg, United Kingdom), Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP, West Boothbay Harbor, ME), the Sammlung von Algenkulturen (SAG) at the University of Göttingen (Göttingen, Germany), and the Culture

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Abbreviations: *psaA*, photosystem I P700 chlorophyll *a* apoprotein A1; *psbA*, photosystem II reaction center protein D1; *rbcl*, ribulose-1,5-bisphosphate carboxylase/oxygenase; ME, minimum evolution; LgD, LogDet; MCMC, Markov chain Monte Carlo; SH, Shimodaira-Hasegawa.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY119693–AY119789).

See commentary on page 11558.

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Collection of Algae at the University of Texas at Austin (UTEX). Some of Cyanidiales red algae were collected in the field and maintained at the Dipartimento di Biologia Vegetale (DBV) culture collection at the University of Naples, Italy. *Chondrus crispus* and *Palmaria palmata* were collected from Nova Scotia and Maine. The species, strain numbers and collection sites of these taxa are listed in Table 1, which is published as supporting information on the PNAS web site, www.pnas.org. The algal cultures were frozen in liquid nitrogen, and ground with glass beads by using a glass rod and/or MiniBeadBeater (Biospec Products, Bartlesville, OK). Total genomic DNA was extracted by using the DNeasy Plant Mini kit (Qiagen, Valencia, CA). PCRs were done by using specific primers for each of the plastid genes (Table 2, which is published as supporting information on the PNAS web site). The presence of highly variable third codon positions in the *psaA* gene led us to use species-specific primers based on sequences in sister species. Because introns were found in the *psaA* gene of some red algae, the reverse transcription (RT)-PCR method was used to isolate cDNA. For the RT-PCR, total RNA was extracted by using the RNeasy Mini kit (Qiagen). To synthesize cDNA from total RNA, Moloney murine leukemia virus Reverse Transcriptase (GIBCO/BRL) was used following the manufacturer's protocol. PCR products were purified by using the QIAquick PCR Purification kit (Qiagen), and were used for direct sequencing with the BigDye Terminator Cycle Sequencing kit (Applied Biosystems), and an ABI-3100 at the Center for Comparative Genomics at the University of Iowa. Some PCR products were cloned into pGEM-T vector (Promega) before sequencing.

Phylogenetic Analyses. Sequences were manually aligned by using SEQUP (21). The data sets used in the phylogenetic analyses are available from D.B. In the first analysis, we used a collection of concatenated *psaA* and *psbA* genes that contained 22 rhodophytes, 4 cryptophytes, 7 haptophytes, 4 stramenopiles, 7 dinoflagellates, 2 chlorophytes, and a glaucophyte as the outgroup. In the second analysis of a *psbA* data set, we added 8 peridinin-containing dinoflagellates, 3 stramenopiles, and 1 cryptophyte. In the third data set of *rbcL* sequences, we used 40 taxa that contained the "red-type Form I" *rbcL* gene (i.e., excluding the chlorophyte, glaucophyte, and peridinin-containing dinoflagellates, see ref. 22), with the Cyanidiales red algae as the outgroup. Trees were inferred with the minimum evolution (ME) method using LogDet (ME-LgD) distances (23) and the PAUP*4.0b8 (24) computer program. Ten heuristic searches with random-addition-sequence starting trees and tree bisection and reconnection branch rearrangements were done to find the optimal ME tree. To test the stability of monophyletic groups in the ME tree, 2,000 bootstrap replicates were analyzed (25) with the DNA (LogDet distance) and protein (Poisson corrected distances, MEGA v2.0; ref. 26) data sets (ME-Pr). We also conducted Bayesian analysis of the DNA data (MRBAYES v2.0; Ba-D; ref. 27) using a general time reversible (GTR) model and a site-specific γ parameter for each codon site. Bayesian posterior probabilities are roughly equivalent to maximum likelihood bootstrap analysis (28, 29). Markov chain Monte Carlo (MCMC) from a random starting tree was initiated in the Bayesian inference and run for 500,000 generations. A consensus tree was made with the MCMC trees after convergence. For the *rbcL* data, the GTR + I + Γ model was used in the Bayesian inference using only first + second codon positions (810 nt).

The Shimodaira-Hasegawa (SH) nonparametric bootstrap test was used to compare alternative phylogenetic hypotheses regarding the position of peridinin- and fucoxanthin-containing dinoflagellates (30). The SH test was done by using PAUP*4.0b8, with REL (resampling estimated log-likelihood) optimization, and 100,000 bootstrap replicates.

Results

PsaA + PsbA Phylogeny. A total 2,352 nucleotides and 784 amino acids from 47 taxa were used in the analysis of the *psaA* + *psbA* data set. The ME-LgD tree of the concatenated sequences shows strong support for the monophyly of fucoxanthin- and peridinin-containing dinoflagellates (ME-LgD = 99%, ME-Pr = 99%, Fig. 1A). The dinoflagellates are positioned as sister to the haptophytes with robust bootstrap support and a significant Bayesian posterior probability for this node (ME-LgD = 100%, ME-Pr = 77%, Ba-D = 1.0). Use of only first and second, or only the most highly conserved second codon positions of the *psaA* + *psbA* data set in ME-LgD analyses also recovered monophyly of fucoxanthin- and peridinin-containing dinoflagellate plastids (results not shown). In Fig. 1A, *P. foliaceum* is nested within the *Skeletonema* and *Odontella* clade with strong bootstrap and Bayesian support, confirming its origin from a diatom through plastid replacement (17, 18).

We tested alternative hypotheses by using the data set of all three codon positions of *psaA* + *psbA* and the SH test (Fig. 1B). In these analyses, the paraphyly of the fucoxanthin-containing dinoflagellates was not rejected (Fig. 1B, tree 1; $P = 0.296$), whereas the monophyly of peridinin-containing and rhodophyte plastids (the conventional hypothesis; Fig. 1B, tree 2; $P < 0.000$) and stramenopiles plastids (Fig. 1B, tree 3; $P = 0.004$) was resoundingly rejected. Forcing two independent origins of peridinin in the plastids of *Amphidinium* and *Heterocapsa* resulted in a significantly worse tree (Fig. 1B, trees 4 and 5), suggesting that peridinin had a single origin as shown in Fig. 1A.

PsbA Phylogeny. The ME-LgD tree of *psbA* sequences, which was inferred from a data set of 957 nucleotides and 319 amino acids from 59 taxa, shows a very similar topology to the *psaA* + *psbA* phylogeny. The dinoflagellates form a monophyletic clade with haptophytes (Fig. 2A). The dinoflagellates + haptophytes clade is positioned as sister to the stramenopiles, with moderate to strong support in the minimum evolution (ME-Pr = 78%) and Bayesian ($P = 1.0$) analyses. Within the dinoflagellate clade, the 11 peridinin-containing species are monophyletic with strong support (ME-LgD = 88%, ME-Pr = 99%, Ba-D = 1.0), whereas the fucoxanthin-containing species are paraphyletic at the base of this lineage, consistent with the SH test using the *psaA* + *psbA* data (Fig. 1B, tree 1). The peridinin-containing dinoflagellates form two major clades, one that includes *Amphidinium* + *Heterocapsa*, and a second that contains the remainder of the species. Again, use of first and second, or only the second codon positions of the *psbA* data set in ME-LgD analyses showed monophyly of fucoxanthin- and peridinin-containing dinoflagellate plastids (results not shown). As found above, the *P. foliaceum psbA* sequence grouped with the diatoms (i.e., with *Odontella* and *Skeletonema*).

RbcL Phylogeny. The ME-LgD analysis of the first and second codon positions of the "Form I" *rbcL* gene supports the sister group relationship of fucoxanthin-containing dinoflagellate and haptophyte plastids with a significant Bayesian posterior probability ($P = 0.98$; Fig. 2B) for this node (see Fig. 4, which is published as supporting information on the PNAS web site, for the complete tree). The ME-LgD and ME-Pr bootstrap analyses provided, however, only weak support for dinoflagellate-haptophyte monophyly (65% and 31%, respectively). The usage of all three *rbcL* codon positions in the ME-LgD analysis resulted in a phylogeny that did not support the monophyly of fucoxanthin-containing and haptophyte plastids. The branch lengths within the *Karlodinium-Karenia* clade were, however, relatively long compared with the haptophyte *rbcL* sequences when third codon positions were included. This likely explains the "attraction" (31) of the fucoxanthin clade to the outgroup

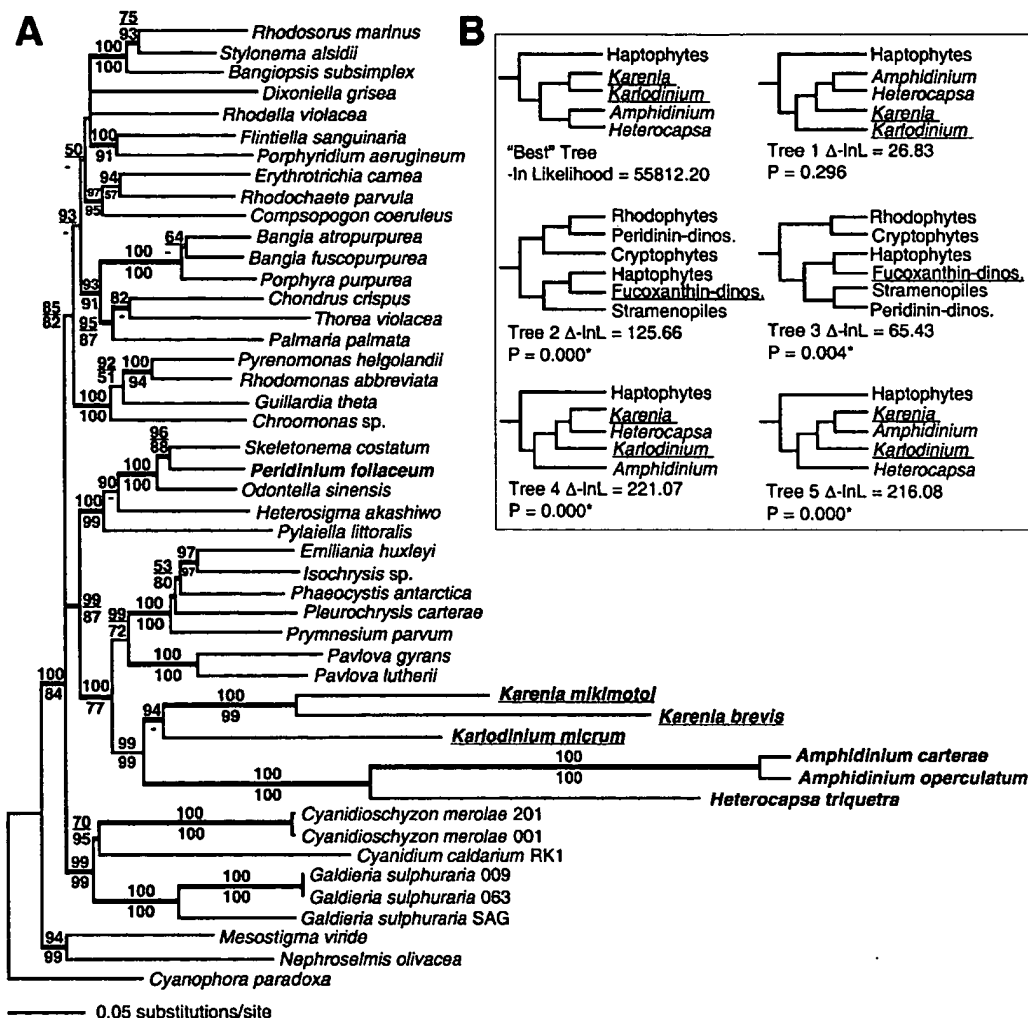


Fig. 1. Phylogeny of red algal and red algal-derived plastids by using the combined *psaA* and *psbA* sequences. (A) Minimum evolution tree using LogDet distances. The bold letters indicate all dinoflagellates, whereas the underlined taxa contain fucoxanthin. A total of 2,352 nucleotides were considered. The LogDet bootstrap values (2,000 replications) are shown above the branches, and ME protein Poisson bootstrap values are shown below the branches. The thick branches denote >95% posterior probability for groups to the right resulting from a Bayesian inference. A total of 500,000 MCMC generations were run, and the posterior probabilities were determined from 4,368 probable trees. (B) Comparison of the best ME-LgD tree to alternative topologies by using the nonparametric SH bootstrap test. The best tree favored a sister group relationship of peridinin- and fucoxanthin-containing dinoflagellate plastids, however, not significantly (tree 1). The monophyly of dinoflagellate and haptophyte plastid (trees 2 and 3), and the single origin of peridinin-containing dinoflagellate is, however, significantly supported (trees 4 and 5).

Cyanidiales in this tree (results not shown). In support of this hypothesis, use of γ -corrected distances (ME-GTR + I + Γ) recovered the monophyly of fucoxanthin-containing and haptophyte plastids and Bayesian inference by using the *rbcL* amino acid sequences and the JTT + Γ model showed a significant posterior probability for the node uniting fucoxanthin-containing and haptophyte plastids ($P = 1.0$; results not shown). These data provide strong support, therefore, for the monophyly of fucoxanthin-containing and haptophyte plastids. Our results, using an expanded data set of *rbcL* sequences, is consistent with previous reports (32). The two genera of fucoxanthin-containing dinoflagellates, *Karenia* and *Karlodinium*, are paraphyletic at the base of haptophyte clade, a result that is also found in the *psbA* tree.

Additional Analyses. We did ME-LgD and Bayesian analyses that included published plastid 16S rRNA data, by using the most

highly conserved regions of this gene (678 nucleotides). A multigene tree of the concatenated sequences of 16S rRNA + *psaA* + *psbA* (3,030 nucleotides) that included 3 fucoxanthin- and a peridinin-containing dinoflagellate (*Heterocapsa triquetra*) showed significant support for the sister group relationship of dinoflagellates and haptophytes (ME-LgD = 98%, Ba-D = 0.98; see Fig. 5, which is published as supporting information on the PNAS web site). The ME-LgD tree using *psaA* alone showed a similar topology to the phylogenies inferred from *psbA* alone (Fig. 2A) and from *psaA* + *psbA* (Fig. 1A). There was moderate bootstrap support in the *psaA* protein analysis (ME-Pr = 78%) and poor support in the minimum evolution analyses (ME-LgD = 54%) for the sister group relationship of dinoflagellates and haptophytes (results not shown).

We also tested for mutational saturation in the data sets used for phylogenetic analyses by correcting for multiple substitutions using the HKY85 model (DNA) and JTT model (protein).

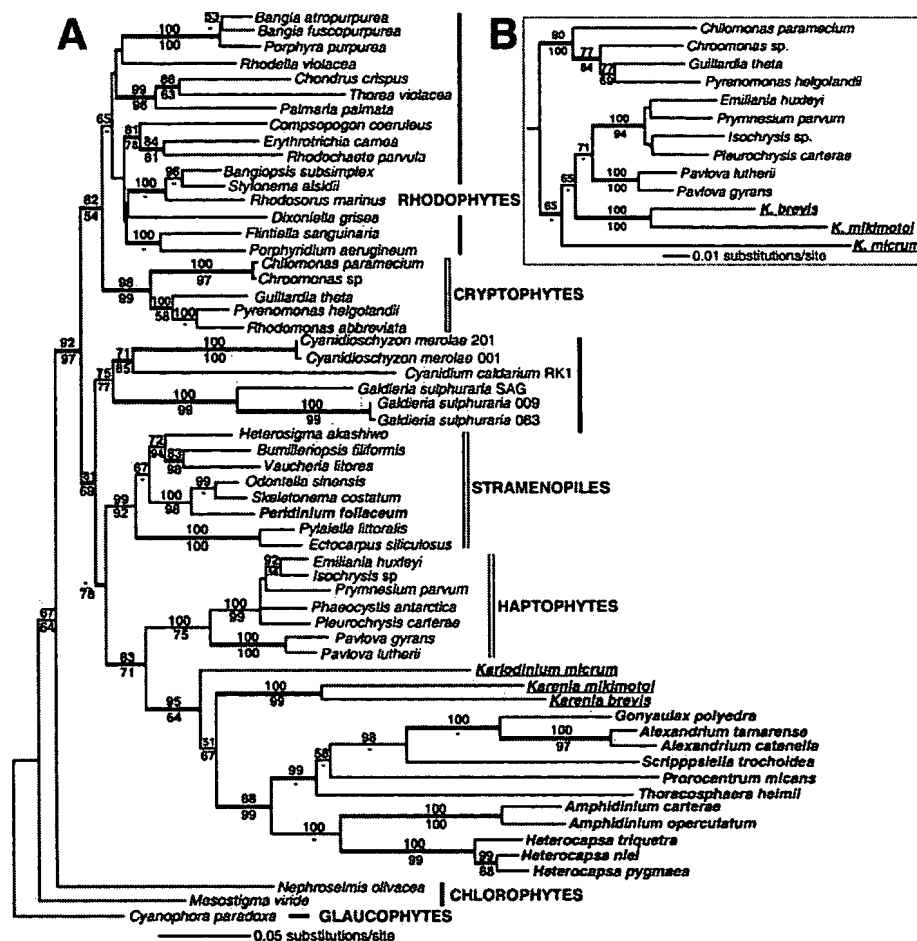


Fig. 2. Phylogeny of red algal and red algal-derived plastids using *psbA* and *rbcL* sequences. (A) Minimum evolution *psbA* tree using LogDet distances. The bold letters indicate all dinoflagellates, whereas the underlined taxa contain fucoxanthin. A total of 957 nucleotides were considered. The LogDet bootstrap values (2,000 replications) are shown above the branches and ME protein Poisson bootstrap values are shown below the branches. The thick branches denote >95% posterior probability for groups to the right resulting from a Bayesian inference. A total of 500,000 MCMC generations were run and the posterior probabilities were determined from 4,141 probable trees. (B) Minimum evolution *rbcL* tree using LogDet distances. Only the subtree containing the fucoxanthin-containing dinoflagellates (underlined) is shown. The entire tree is shown as Fig. 4. A total of 810 nucleotides (only first and second positions) were considered. The bootstrap and Bayesian inference (4,593 probable trees) were done as above.

Corrected versus uncorrected distances were plotted for the *psaA* + *psbA* DNA data set using all three positions, and for the *psaA* + *psbA* protein data (Fig. 6A, which is published as supporting information on the PNAS web site). This analysis showed minimal mutational saturation, although the DNA data showed slightly more saturation among distantly related taxa than did the protein data. We also analyzed first + second versus third codon positions for these genes (Fig. 6B). The first + second positions have a nearly linear relationship over much of the data set, whereas third positions show considerable saturation for highly divergent taxa suggesting that these data are less useful for resolving deep evolutionary relationships (Fig. 6B).

Discussion

Tertiary Haptophyte Plastid Replacement. Taken together, our data provide strong evidence for a common origin of peridinin- and fucoxanthin-containing dinoflagellate plastids. The monophyly of dinoflagellate and haptophyte plastid sequences is recovered in all of our phylogenies (except for the *rbcL* ME-LgD tree using all 3 codon positions; see Results); *psaA* + *psbA* (Fig. 1A), *psbA* (Fig. 2A), and in the 16S rRNA + *psaA* + *psbA* analyses (Fig.

5). Other plastid replacements in the dinoflagellates are, however, clearly more recent events in the peridinin-containing taxa, such as the diatom replacement in *P. foliaceum* (refs. 17 and 18; see Figs. 1A and 2A) and the cryptophyte replacement in *Dinophysis acuminata* (J.D.H., L. Maranda, H.S.Y., and D.B., unpublished data). Our surprising result in this study contradicts the conventional view that only the fucoxanthin-containing taxa underwent a haptophyte plastid replacement (e.g., refs. 2, 11, and 15) and provides a paradigm for understanding dinoflagellate plastid evolution. Based on our results and the accepted secondary endosymbiotic origin of the haptophyte plastid (e.g., ref. 33), we postulate that the ancestral dinoflagellate acquired its plastid from a haptophyte through a tertiary plastid replacement. The SH test shows the haptophyte origin model to have significantly greater support than any of the alternative hypotheses (Fig. 1B, trees 2 and 3), such as a red algal origin (7), or a stramenopiles origin (34). However, we cannot discriminate between a monophyletic or paraphyletic origin of the fucoxanthin-containing dinoflagellates (Fig. 1B). And, as previously shown, our data also support the monophyly of peridinin-containing dinoflagellates (11, 20).

One realistic concern about our results is that they may be an artifact of the relatively high divergence rate of dinoflagellate plastid genes rather than reflecting the true evolutionary history of the organelles. In this scenario, the dinoflagellate sequences may be “attracted” together because they share long branches rather than because of a monophyletic origin (31). Previous analyses (e.g., refs. 7 and 11) have clearly been hampered by this problem and the trees have often not provided an unambiguous placement of the dinoflagellate plastids (e.g., figure 5 in ref. 11). We have addressed this potentially confounding problem in the following ways. First, we have increased the taxon sampling for both red algae and their derived plastids to include all potential sister groups for the dinoflagellate genes and to break long branches that may cause homoplasious attraction. Second, we have used robust phylogenetic methods to ameliorate the effects of high divergence rates (i.e., γ -corrected distances, Bayesian inference, use of protein sequences, use of only first and second or only the most highly conserved second codon positions) and a biased nucleotide content (LogDet transformation). Third, we have increased the phylogenetic signal by augmenting the length of the sequences being compared through multigene sampling. The fact that each gene, singly or in combination, supports the monophyly of fucoxanthin- and peridinin-containing dinoflagellate plastids suggests to us that this result is robust and not the outcome of an uniform bias in all of these genes.

The best way to correct for long-branch attraction is to sample slowly evolving genes (35), but this may be an improbable solution for dinoflagellates because all plastid genes that have been studied until now have elevated divergence rates (e.g., refs. 7, 11, and 20). However, our analyses of pairwise sequence distances indicate that the *psaA* and *psbA* coding regions do not show extensive mutational saturation and that even the third positions of these sequences encode phylogenetic signal (see Fig. 6). Use of nuclear-encoded photosynthetic genes would be another possible solution, but we predict that many of these genes would support a red algal ancestry of the dinoflagellate plastid because they trace their origin to the initial secondary endosymbiosis (36). Some plastid-targeted genes of haptophyte origin are also predicted to exist in the dinoflagellate nuclear genome as a result of gene transfer following the tertiary endosymbiosis. Analysis of plastid genes is, therefore, of fundamental importance to understanding plastid evolution in the dinoflagellates.

Given that our hypothesis of the monophyly of fucoxanthin- and peridinin-containing dinoflagellate plastids is correct, then we envision two possible scenarios for their relative order of divergence: (i) after the tertiary haptophytic-plastid replacement, the fucoxanthin- and peridinin-containing dinoflagellates diverged as sister groups (Fig. 1A), or (ii) the peridinin-containing dinoflagellates emerged from within a clade of fucoxanthin-containing ancestors (Fig. 2A). In either case, we hypothesize that the fucoxanthin-containing plastid (13) should be regarded as the primitive condition (i.e., fucoxanthin is present in the plastid donor) with the presence of peridinin being a derived state. This idea is reinforced by the observation that peridinin-containing dinoflagellate plastids also share a suite of unique characters not found in fucoxanthin plastids (e.g., “Form II” *rbcL* gene, single-gene circles; see below), which strongly supports a single origin and monophyly of these taxa. However, our data do not allow us to determine the timing of the haptophyte replacement. It may have occurred at the base of the dinoflagellates or this tertiary plastid may have a much longer evolutionary history.

A fully resolved host tree of the dinoflagellates is critical to test our hypothesis of a basal haptophyte replacement. Presumably, the topology of this tree should mirror that of the plastid trees given a single organelle origin. Existing analyses using nuclear small subunit rRNA show the fucoxanthin-containing species as

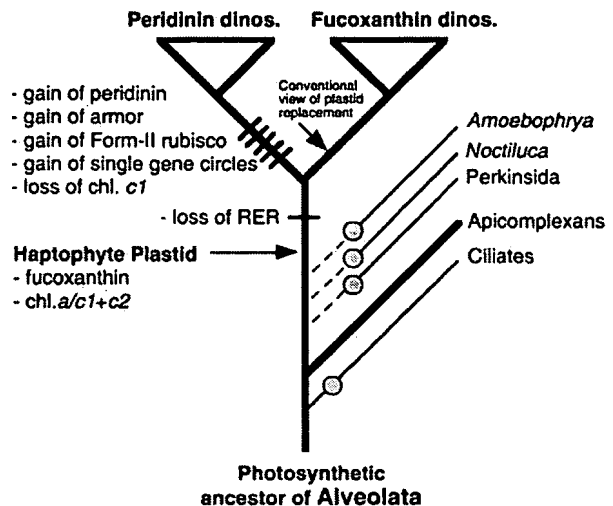


Fig. 3. Putative model of plastid evolution mapped on a current “host” tree of the Alveolata (adapted from Saldarriaga *et al.*; ref. 10). Plastid-containing lineages are shown with the thick branches and plastid loss is denoted with a filled circle. The ancestral alveolate plastid is presumed to have been lost multiple independent times in the aplastidial taxa (i.e., Perkinsida, Noctiluca, and Amoeboophrya). The broken lines denote uncertainty about the evolutionary interrelationships of the aplastidial taxa. The hatchmarks indicate the origin of novel characters in the peridinin-containing plastids. The haptophyte plastid replacement is believed to have occurred at least before the split of the fucoxanthin- and peridinin-containing dinoflagellates. The conventional view of the haptophyte plastid replacement occurring on the branch uniting fucoxanthin-containing plastids is shown.

an early diverging group, but with no bootstrap support for their monophyly or for their relationship to peridinin-containing taxa (10). Trees inferred with large subunit rRNA support monophyly of fucoxanthin-containing species but with poor support for their position relative to other dinoflagellates (12, 37).

Evolution of Dinoflagellate Plastids. A model of dinoflagellate plastid evolution is presented in Fig. 3. This scheme presumes that the common ancestor of the alveolates contained a plastid of red algal origin (irrespective of whether it was a secondary or a tertiary endosymbiont; refs. 10, 34, and 36). This plastid was apparently lost in the ciliates, whereas in the Apicomplexa, which are intracellular parasites, it was reduced to a nonphotosynthetic organelle of unknown function (38). The branching of several nonphotosynthetic lineages before the divergence of the photosynthetic dinoflagellates suggests that multiple plastid losses may have occurred (i.e., Perkinsida, Noctiluca, and Amoeboophrya; ref. 10) in the evolution of the alveolates. Some time after the haptophyte replacement of the plastid, the dinoflagellates diverged into two groups. One group retained the ancestral haptophyte characteristics in its plastid (fucoxanthin, chlorophyll $c_1 + c_2$), whereas the second group underwent several major changes including the evolution of peridinin (replacing fucoxanthin), loss of chlorophyll c_1 , the remarkable reduction of its plastid genome to single gene circles (7, 39), the development of cellulose armor, and the origin of a divergent nuclear encoded “Form II” *rbcL* gene (40, 41). It is possible that peridinin could have evolved from a modification of the ancestral fucoxanthin biosynthesis pathway because these are structurally related xanthophylls (for details, see refs. 13 and 42). Because haptophytes contain a 4-membrane bound plastid, we postulate that the outer rough endoplasmic reticulum membrane of this organelle was lost after the tertiary endosymbiosis resulting in the 3 smooth

membranes that surround both fucoxanthin- and peridinin-containing dinoflagellate plastids (14).

In summary, our results provide important insights into the complex evolutionary history of dinoflagellates. Previous analysis of nuclear-encoded cytosolic genes have suggested a sister group relationship to the stramenopiles, indicating the host affinity for this group (43, 44). Nuclear-encoded, photosynthetic genes suggest an origin of the plastid from a red alga (34, 36). These genes have presumably been inherited vertically from the common ancestor of stramenopiles and dinoflagellates (i.e., the shared red algal secondary endosymbiont, refs. 34 and 36; Fig. 3). Plastid-encoded genes of both peridinin- and fucoxanthin-containing dinoflagellates show a strong relationship to the haptophytes, indicating that a tertiary plastid replacement occurred before the split of these lineages. As stated above, our

model predicts the presence of plastid-targeted, nuclear-encoded genes (and possibly cytosolic genes) of both red algal and haptophyte origin in the dinoflagellate nucleus. A genome-wide approach will most likely be necessary to understand fully the relative contributions of these successive endosymbioses to dinoflagellate evolution. Most remarkably, some of the peridinin-containing taxa have gone on to replace their plastid yet again [e.g., *P. foliaceum* (17, 18) and *Dinophysis* (16)]. The genomic contribution of these additional rounds of endosymbiosis to dinoflagellate evolution also remains to be determined.

We thank D. W. Freshwater and E. H. Bae for providing dried thallus of *Palmaria* and *Chondrus*. H.S.Y. was supported by the Post Doctoral Fellowship Program of the Korean Science and Engineering Foundation. This work was supported by National Science Foundation Grants DEB 01-07754 and MCB 01-10252 (to D.B.).

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Table 1. Sample information and GenBank accession numbers for taxa included in phylogenetic analyses

Taxa	Source	GenBank accession nos.		
		<i>psaA</i>	<i>psbA</i>	<i>rbcL</i>
Rhodophyte				
<i>Cyanidioschyzon merolae</i>	DBV 201 JAVA	AY119693	AY119729	AY119765
	DBV 001 NAPS	AY119694	AY119730	AY119766
<i>Cyanidium caldarium</i>	RK1	AF022186	AF022186	AF022186
<i>Galdieria sulphuraria</i>	SAG 108.79	AY119695	AY119731	AY119767
	DBV 009 VTNE	AY119696	AY119732	AY119768
	DBV 063 AGCS	AY119697	AY119733	AY119769
<i>Bangia atropurpurea</i>	SAG 33.94	AY119698	AY119734	AY119770
<i>Bangia fuscopurpurea</i>	SAG 59.81	AY119699	AY119735	AY119771

<i>Bangiopsis subsimplex</i>	PR21	AY119700	AY119736	AY119772
<i>Compsopogon coeruleus</i>	SAG 36.94	AY119701	AY119737	AF087116
<i>Dixoniella grisea</i>	SAG 39.94	AY119702	AY119738	AY119773
<i>Erythrotrichia carnea</i>	UTEX LB 1425	AY119703	AY119739	AF087118
<i>Flintiella sanguinaria</i>	SAG 40.94	AY119704	AY119740	AY119774
<i>Porphyra purpurea</i>	GenBank	U38804	U38804	U38804
<i>Porphyridium aerugineum</i>	SAG 1380-2	AY119705	AY119741	AY119775
<i>Rhodella violacea</i>	SAG 115.79	AY119706	AY119742	AY119776
<i>Rhodochaete parvula</i>	UTEX LB 2715	AY119707	AY119743	AY119777
<i>Rhodorus marinus</i>	SAG 116.79	AY119708	AY119744	AY119778
<i>Stylonema alsidii</i>	SAG 2.94	AY119709	AY119745	AY119779
<i>Chondrus crispus</i>	Nova Scotia, Canada	AY119710	AY119746	U02984
<i>Palmaria palmata</i>	Maine, USA	AY119711	U28165	U28421
<i>Thorea violacea</i>	SAG 51.94	AY119712	AY119747	AF029160
Cryptophyte				
<i>Chilomonas paramecium</i>	SAG 977.2a	Missing	AY119748	AY119780
<i>Chroomonas</i> sp.	SAG 980-1	AY119713	AY119749	AY119781
<i>Guillardia theta</i>	GenBank	AF041468	AF041468	AF041468
<i>Pyrenomonas helgolandii</i>	SAG 28.87	AY119714	AY119750	AY119782
<i>Rhodomonas abbreviata</i>	CCAP 979/16	AY119715	AY119751	–
Haptophyte				
<i>Emiliania huxleyi</i>	SAG 33.90	AY119716	AY119752	AB043631

<i>Isochrysis</i> sp.	SAG 927-2	AY119717	AY119753	AY119783
<i>Pavlova gyraus</i>	CCMP 607	AY119718	AY119754	AY119784
<i>Pavlova lutherii</i>	CCMP 1325	AY119719	AY119755	AY119785
<i>Phaeocystis antarctica</i>	CCAP 943/5	AY119720	AY119756	–
<i>Pleurochrysis carterae</i>	CCAP 961/1	AY119721	AY119757	D11140
<i>Prymnesium parvum</i>	UTEX LB 995	AY119722	AY119758	AB043698
Stramenopiles				
<i>Heterosigma akashiwo</i>	CCMP 1595	AY119723	AY119759	X61918
<i>Odontella sinensis</i>	GenBank	Z67753	Z67753	Z67753
<i>Pylaiella littoralis</i>	CCMP 1907	AY119724	AY119760	X55372
<i>Skeletonema costatum</i>	CCMP 1332	AY119725	AY119761	AF015569
<i>Bumilleriopsis filiformis</i>	SAG 809-2	–	X79223	–
<i>Ectocarpus siliculosus</i>	GenBank	–	X56695	–
<i>Vaucheria litorea</i>	GenBank	–	AF227740	–
Dinoflagellate				
<i>Karenia brevis</i>	CCMP 718	AY119726	AY119762	AY119786
<i>Karlodinium micrum</i>	CCMP 415	AY119727	AY119763	AY119787
<i>Peridinium foliaceum</i>	CCMP 1326	AY119728	AY119764	U31876
<i>Karenia mikimotoi</i>	G303ax-2	AB027235	AB027234	AB034635
<i>Heterocapsa triquetra</i>	CCMP 449	AF130031	AF130033	–
<i>Amphidinium operculatum</i>	CCAP 1102/6	AJ250264	AJ250262	–
<i>Amphidinium</i>	CS-21	AJ311631	AF206672	–

<i>carterae</i>				
<i>Alexandrium catenella</i>	TN7	–	AB025590	–
<i>Alexandrium tamarense</i>	OF151	–	AB025589	–
<i>Gonyaulax polyedra</i>	GenBank	–	AB025588	–
<i>Heterocapsa niei</i>	CCMP 447	–	AF206709	–
<i>Heterocapsa pygmaea</i>	CCMP 1490	–	AF206707	–
<i>Prorocentrum micans</i>	NIES-12	–	AB025585	–
<i>Scrippsiella trochoidea</i>	NEPCC D602	–	AF206710	–
<i>Thoracosphaera heimii</i>	NEPCC D670	–	AF206712	–
Chlorophyte				
<i>Mesostigma viride</i>	NIES-296	AF166114	AF166114	–
<i>Nephroselmis olivacea</i>	NIES 484	AF137379	AF137379	–
Glaucophyte				
<i>Cyanophora paradoxa</i>	UTEX LB555	U30821	U30821	–

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Genes	Name	Primer Sequences (5'-3')	Specificity
<i>psaA</i>	psaA130F	AACWACWACTTGGATTGGA	General
	psaA180F	GATAGTCAWACHAGTTCWTTAGA	General
	psaA840F	TTYAAAGGWGGNYTAAAYCC	General
	psaA870F	GGNGGWYTATGGTTAAGTGA	General
	psaA930R	CCCAATTAGTTCTATACATRT	General
	psaA940R	CTGTGDCCAATACCCARTT	General
	psaA970R	GCYTCTARAATYTCTTTCA	General
	psaA1600R	GCATGAATATGRTGWACCAT	General
	psaA1760R	CCTCTWCCWGGWCCATCRCAWGG	General
	psaA-3'	CCTTGAGTDATRCTTAAAGCTC	General
	Crypto230F	AGTGCACATTTTGGWCAATT	Cryptophyte
	Crypto930F	GCTGGTCAYATGTATCGTAC	Cryptophyte
	Crypto1190R	CCACCRATCCACATATGATG	Cryptophyte
	Palm754F	CTAATGGTTCAGTTGTACCC	<i>Palmaria</i>

	Palm1180F	ACACATCATATGTGGATTGG	<i>Palmaria</i>
	Pav940F	ATGTACCGTACTAAYTGGGG	<i>Pavlova</i>
	Flin950F	CGTACTAATTGGGGCATTGG	<i>Flintiella</i>
<i>psbA</i>	psbA-F	ATGACTGCTACTTTAGAAAGACG	General
	psbA500F	CTCTGATGGWATGCCWYTAGG	General
	psbA600R	CCAAATACACCAGCAACACC	General
	psbA-R2	TCATGCATWACTTCCATACCTA	General
	psbA-R1	GCTAAATCTARWGGGAAGTTGTG	General
<i>rbcL</i>	F-rbcL start*	TGTGTTGTCGACATGTCTAACTCTGTAGAAG	General
	rbcL090F	CCATATGCYAAAATGGGATATTGG	General
	F160 [†]	CCTCAACCAGGAGTAGATCC	General
	rbcL170F	CCWCAACCAGGTGTTGAY	General
	rbcL330F	TTTGCTTAYATYGCWTACGA	General
	F-577*	GTATATGAAGGTCTAAAAGGTGG	General
	rbcL640F	ATGATGAAAAYATTAATTCTCAACC	General
	rbcL770R	ATACATTTCTTCCATAGTTGC	General
	R-753*	GCTCTTTCATACATATCTTCC	Bangiaceae
	rbcL1240R	TGWCCRATDGTACCACCACC	General
	rbcL-R [†]	ACATTTGCTGTTGGAGTCTC	General
	R-rbcS start*	TGTGTTGCGGCCGCCCTTGTGTTAGTCTCAC	General

*Freshwater, D. W. & Rueness, J. (1994) *Phycologia* **33**, 187–194.

[†]Rintoul, T. L., Sheath, R. G. & Vis, M. L. (1999) *Phycologia* **38**, 517–527.

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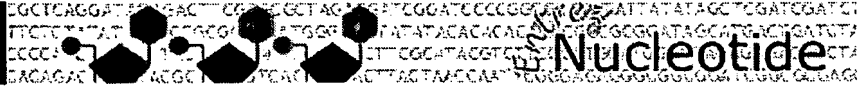
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Display Show Send to Hide: ☐ sequence ☐ all but gene, CDS and mRNA features

Range: from to ☐ Reverse complemented strand Features:

☐ 1: [AY119786](#). Reports *Karenia brevis* ri...[gi:21913674]

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LOCUS AY119786 907 bp DNA linear PLN 05-SEP-2002
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 VERSION AY119786.1 GI:21913674
 KEYWORDS .
 SOURCE chloroplast *Karenia brevis*
 ORGANISM [Karenia brevis](#)
 Eukaryota; Alveolata; Dinophyceae; Gymnodiniales; Gymnodiniaceae; *Karenia*.
 REFERENCE 1 (bases 1 to 907)
 AUTHORS Yoon,H.S., Hackett,J.D. and Bhattacharya,D.
 TITLE A single origin of the peridinin- and fucoxanthin-containing plastids in dinoflagellates through tertiary endosymbiosis
 JOURNAL Proc. Natl. Acad. Sci. U.S.A. 99 (18), 11724-11729 (2002)
 PUBMED [12172008](#)
 REFERENCE 2 (bases 1 to 907)
 AUTHORS Yoon,H.S., Hackett,J.D. and Bhattacharya,D.
 TITLE Direct Submission
 JOURNAL Submitted (10-JUN-2002) Department of Biological Sciences and Center for Comparative Genomics, University of Iowa, 239 Biology Building, Iowa City, IA 52242, USA
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 IIVMIDLVIQSMAYWARNDTMLHLHRAGNSTYSRQKNHGMNFRVICKWMRMA
 GVDHIHAGTVVGKLEGPDKMIKGFYETLLGLCTRINLSLGLFFGQEWASLRKCV
 PVASGGIHAGQMHYLLHYLGDDVILQFGGG"
 ORIGIN
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 61 aatatttttg gttttaaacg tgtaaaagtct cttcggttg aggatatgag aatcccggtg
 121 gcttatctaa aaacgtttca aggtccagct actggtttag tcgttgaacg agaacgtcta
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721 gggttctatg aaacgttatt gggctctgtg acacgaatta accttagtct cgggttattt
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901 ggtggta
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Exhibit E

CCMP718 <i>Karenia brevis</i> (Davis) Hansen et Moestrup -- Class Dinophyceae			
Collected: -	Isolated by: Wilson		
Collection Site: 27.7000N 82.8000W Florida USA (very approx.)	Isolation Date: 1960's		
Ocean: North Atlantic	Identified by:		
Sea: Caribbean Sea????	Deposited by: Tangen,K		
Nearest Continent: North America	Deposit Date: 1985-06-13		
Other Information:	Axenic by:		
	Axenic Date:		
	Axenic (True/False): False		
Culture medium: L1, f/2-Si, f/20-Si, f/2, K	Strain Synonyms: PTBR		
Temp. Range at CCMP: 18-22° C	Name Gymnodinium breve,		
Cell length: (Observed) 24-36µm	Synonyms: Ptychodiscus breve		
Cell width: at CCMP) 5-7µm			
Toxic (True only): Yes			

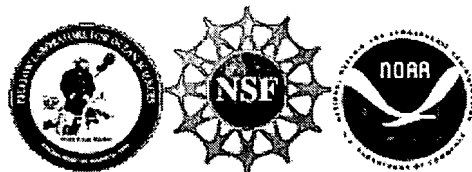


Exhibit F

Research Report

Design Strategies and Performance of Custom DNA Sequencing Primers

BioTechniques 27:528-536 (September 1999)

Research Report

Design Strategies and Performance of Custom DNA Sequencing Primers

G.A. Buck¹, J.W. Fox², M. Gunthorpe³, K.M. Hager⁴, C.W. Naeve⁵, R.T. Pon⁶, P.S. Adams⁷, and J.R. Rush⁸

¹Virginia Commonwealth University, Richmond, VA; ²University of Virginia, Charlottesville, VA; ³University of California, San Francisco, CA; ⁴Yale University, New Haven, CT; ⁵St. Jude Children's Research Hospital, Memphis, TN, USA; ⁶University of Calgary, Calgary, AB, Canada; ⁷Trudeau Institute, Saranac Lake, NY and ⁸Harvard Medical School, Boston, MA, USA

ABSTRACT

This study surveyed strategies of sequencing primer selection and evaluated primer performance in automated DNA sequencing. We asked participants to relate their preferred primer design strategies to identify primer characteristics that are considered most important in sequencing primer design. The participants preferred primers of 18-24 nucleotides (nt), 39%-58% G+C, a melting temperature (T_m) of 33-65°C with a 1-2 nt 3'-GC clamp, hairpin stems of less than 2-3 bp, homopolymeric runs of less than 4-5 nt, primer dimers of less than 3-4 bp, and secondary priming sites of less than 3-4 bp. We provided a 300-bp test sequence and asked participants to submit sequences of 1-3 optimal sequencing primers. Submitted

primers ranged from 17-24 nt and largely conformed to the preferred parameters. Submitted primers were distributed across the test sequence, although some sites were disfavored. Surprisingly, approximately 45% of the primers were selected "manually", more than by any software package. Each of 69 submitted and 95 control primers, distributed at 3-bp intervals across the test sequence, were synthesized, purified and tested using a Model 377 PrismTM DNA Sequencer with dichlororhodamine dye terminator reagents (dRhodamine dye terminators). Approximately half of the control primers were also tested using rhodamine dye terminator reagents ("old" rhodamine dye terminators). The results indicated that primer physico-chemical characteristics thought to have a strong impact on sequencing performance had surprisingly little effect. Thus, primers with high or low percent G+C or 1-2 nt strong secondary priming scores or long 3' homopolymeric stretches yielded excellent sequences with the dRhodamine dye terminator reagents, although each of the submitted and 95 control primers had a stronger effect when the old rhodamine reagents were used. The old rhodamine reagents gave sequences with a similar average read length, but the number of errors and ambiguities or "N's" was consistently higher. Moreover, the effects of the primer physico-chemical characteristics were also more evident with the old rhodamine dyes. We conclude that under optimal sequencing conditions with highly pure template and primer, many of the commonly applied primer design parameters are dispensable, particularly when using one of the new generation of sequencing reagents such as the dichlororhodamine dye terminators.

INTRODUCTION

The Nucleic Acids Research Committee (NARC) of the Association of Biomolecular Resource Facilities (ABRF) evaluates the procedures and performance of research facilities that perform nucleic acid synthesis and sequencing in academic, industrial and other institutions (5,6). These studies provide a measure of the industry state-of-the-art, as well as the level of performance, that can be expected from such facilities. Previous projects have examined general operations of DNA core laboratories (15), the accuracy of automated DNA sequencing in these facilities (12), and the performance of unpurified synthetic oligonucleotides as primers for automated DNA sequence analysis (14). The DNA Sequencing Research Committee of the ABRF has performed studies evaluating the ability of DNA core facilities to sequence difficult templates (2) and the performance of various sequencing strategies and chemistries (1). Herein we have studied primer selection strategies used by DNA sequencing facilities and empirically examined the quality of the sequence data provided by primers selected by these facilities.

Despite a long-standing conviction within the DNA sequencing community that careful primer design is essential to ensure high quality data (cf. 11,14,17,18), there is a paucity of empirical studies supporting this view. A variety of reasonable "rules of thumb" (13) suggesting optimal ranges for primer length, percent G+C, melting

Table 1. Predicted Optimal and Observed Physico-Chemical Characteristics of Primers for Automated DNA Sequencing or PCR

	Sequencing Primers ^a		PCR Primers ^b		Submitted Primers ^c	
	Optimum ^d	Range ^e	Optimum ^d	Range ^e	Mean ^f	Range ^g
Length	18–24 nt	15–40 nt	18–29 nt	15–40 nt	19.7 nt	16–24 nt
T _m	52°–88°C	40°–95°C	50°–69°C	40°–95°C	66°C	48°–91°C
Percent G+C of primer	40%–61%	30%–70%	40%–61%	30%–70%	53%	29%–82%
3' GC clamp	1–2 nt	0–5 nt	1–2 nt	0–2 nt	1.1 nt	0–4 nt
Primer dimer	3–4 bp	0–7 bp	3–4 bp	0–7 bp	5.4	1.6–16.5
Hairpin stems	2–3 bp	0–6 bp	2–3 bp	0–6 bp	1.1 bp	0–5 bp
Homopolymer runs	4–5 nt	3–10 nt	4–5 nt	3–10 nt	3.0 nt	2–5 nt
Secondary priming ^m	3–4	0–10 bp	3–4 bp	0–10 bp	49n	0–266n

^aParameter values preferred by participants for primers to be used in sequencing.
^bParameter values preferred for primers to be used in PCR experiments.
^cParameter values of the primers submitted by participants.
^dOptimum values for each parameter as selected by the participants.
^eThe range of acceptable values selected by the participants.
^fThe mean values for each parameter calculated from the submitted primers.
^gThe range of values for each parameter calculated from the submitted primers.
^hValues submitted by participants for primers to be used for sequencing or PCR and nearest neighbor T_m of primers submitted by participants.
ⁱThe 3' clamp refers to the number of G or C bases at the 3' terminus of the primer.
^jLargest number of contiguous complementary bases permitting dimerization of primer or the stability of strongest potential primer dimer (Kcal) as measured by Oligo 5.0.
^kLargest number of bases capable of forming a hairpin stem in the primer.
^lNumber of bases in longest homopolymeric run in the primer.
^mNumber of bases permitted in most stable secondary priming site.
ⁿThe priming efficiency as calculated by Oligo 5.0 for test sequence only (submitted primers).
^oParameter values preferred for primers to be used in PCR experiments.

temperature (T_m) and composition have been used. The submitted primers and a set of control primers spanning the 300-bp test sequence at 3-bp intervals were synthesized and used to sequence the test sequence. However, the success of these strategies in selection of high-quality sequencing primers, to our knowledge, has not been extensively examined. In many sequencing projects, primer design and synthesis represent the most significant costs and consume the bulk of effort and time. Therefore, it is extremely important that these primers yield high-quality sequence data.

To examine this issue, an e-mail survey containing general questions about laboratory functions and specific questions concerning important characteristics for primer selection was distributed. A 300-bp test sequence was provided for which participants were asked to design sequencing primers.

Vol. 27, No. 3 (1999) In many cases, the results of this study, current primer design rules of thumb may be streamlined to facilitate more efficient primer selection for sequencing projects.

MATERIALS AND METHODS

The Survey

In December of 1996 and January of 1997, members of the ABRF were requested via e-mail distributions, the ABRF Electronic Bulletin Board and a standard mailing, to participate in a survey. The survey was distributed via e-mail, the ABRF Electronic Bulletin Board and a standard mailing, to participate in a survey. The survey was distributed via e-mail, the ABRF Electronic Bulletin Board and a standard mailing, to participate in a survey.

study of DNA sequencing primer design. The distribution provided a 300-bp "test sequence" (Figure 1) that was selected and previously shown to contain no segments that affect sequence ladder extension (data not shown). Participants were asked to use the prevailing technology in their laboratories to design and submit the sequences of up to three sequencing primers in the forward direction. Participants were requested to select the best primers irrespective of their positions on the test sequence. All responses were anonymously screened and coded by Virginia Commonwealth University (VCU) Health Sciences Computer Center personnel. Identifying data were removed from each document before being forwarded to the Committee for analysis. Several responses submitted uninterpretable data. Where possible, these responses were identified and excluded from the analysis. The results of this study, current primer design rules of thumb may be streamlined to facilitate more efficient primer selection for sequencing projects.

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fied by Computer Center personnel, and new information was requested. Every effort was made to maintain the integrity of the data and the anonymity of the participants.

Control and Submitted Primers

A panel of 95 primers were synthesized as controls. These 18 nucleotide (nt) primers spanned the 300-bp test sequence with their 5' termini located at 3-bp intervals (i.e., the first began at bp 1 of the test sequence, the second began at bp 4, etc.). All primers were analyzed with the Oligo™ Program 5.0 (NBI/Genovus, Plymouth, MN, USA) (16) and the number of bases, percent G+C, T_m (nearest neighbor method described in Reference 9), internal structure, secondary priming sites, etc., were recorded.

The 69 submitted and 95 control primers were synthesized on a Model 3948 DNA Synthesis and Purification System (PB Biosystems) at three different sites. Synthesis was performed at the 40 nmol scale using PB Biosystems reagents and the standard synthesis, cleavage and purification cycles. An aliquot of each sample was analyzed by polyacrylamide gel electrophoresis (PAGE) and by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF/MS). PAGE was performed using precast minigels (Novex, San Diego, CA, USA) following the protocol specified by the manufacturer. Mass analysis was performed on a 200-pmol sample mixed with 10 mg/mL 3-hydroxytrypsinolonic acid matrix (in 50% acetonitrile, 0.1% trifluoroacetic acid) on a Voyager RP-MALDI-TOF/MS instrument (PerSeptive Biosystems, Framingham, MA, USA). Criteria for passing quality control were: (i) a single major band on the gel; (ii) appropriate mobility on the gel.

compared to molecular weight standards; (iii) a synthesis yield >0.5 ODU (absorption of sample in 1 mL at 260 nm with 1 cm pathlength set up on the 3948 Synthesizer); and (iv) a single peak of the expected mass by MALDI-TOF/MS. Any oligonucleotides that failed to meet these criteria were resynthesized.

Test Template and Preparation

The plasmid template was preselected to contain a test sequence lacking obstacles to sequence extension (data not shown) and purified by double banding in CsCl-ethidium bromide isopycnic density gradients (10).

DNA Sequence Analysis

Each of the purified and quality-tested oligonucleotides were used as a primer for DNA sequence analysis using dRhodamine Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® FS reagents (PB Biosystems) under standard reaction conditions as described by the manufacturer at 20 μ L total vol containing 300 ng of template and 5 pmol of primer. Sequencing reactions were run for 25 cycles of 10 s at 96°C, 5 s at 50°C and 4 min at 60°C in a GeneAmp® PCR System 9700 DNA Thermal Cycler (PB Biosystems). Reactions were analyzed on Model 377 PRISM Automated DNA Sequencers using 5% Long Ranger™ (FMC BioProducts, Rockland, ME, USA) gels with Tris-Borate-EDTA buffer (TBE, pH 8.3). Approximately 30% of the control primers were rerun using the same dRhodamine dye terminator/Taq FS kit to ensure reproducibility, and approximately 50% of the control primers were rerun using the old rhodamine dye terminator/Taq FS kit (PB Biosystems). The dRhodamine or dichlororhodamine

reagents are modifications of the original old rhodamine dyes that have narrower emission spectra, less spectral overlap and more even peak heights than the earlier dyes (19). Each 5% polyacrylamide sequencing gel was electrophoresed for approximately 8 h, and the data was directly exported for analysis using Sequencher™ (Gene Codes, Ann Arbor, MI, USA). Using this software, the raw data was trimmed to remove 5' and 3' ambiguous sequences, so that the first and last 25 nt contained no ambiguities. Each result was aligned with the known sequence, and we recorded: (i) the number of nt between the 3' end of the primer and the beginning of the trimmed data; (ii) the number of nt in each sequence read; and (iii) the number of errors, including miscalls, insertions, deletions and ambiguities in each 100-bp window through the end of the sequence read.

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RESULTS

Participant Profiles

Thirty-nine laboratories participated in the study, submitting 69 primer sequences. Of the participants, approximately 77% provide synthesis services and approximately 85% provide sequencing services, but only approximately 50% provide primer design or walking services, and less than one third offer template preparation. Laboratories performing these services averaged annually approximately 3700 syntheses, 7000 sequence runs, 425 primer designs, 424 template purifications and less than 200 primer walking projects. Charges for services averaged \$1.17/nt for 40–50 nmol and \$2.19/nt for 150–200 nmol syntheses of a 25-nt unpurified primer and \$24/reaction for DNA sequence analysis (89% for gel only). The average price for a primer walking sequencing project of a 2-kbp insert in a plasmid vector was approximately \$500 or \$0.25/bp. Charges for external users, where available, averaged approximately 17%–50% higher than charges for institutional users. DNA synthesis and sequencing instrumentation was dominated by PB Biosystems, i.e., 77% of synthesizers and 92% of sequencers, but Beckman

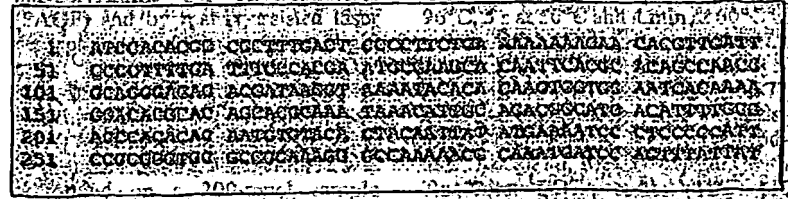


Figure 1. Test sequence. Shown is the 300-bp sequence distributed to participants for design of forward sequencing primers. Participants were asked to design primers in the forward direction.

Instruments, PerSeptive Biosystems and LI-COR were also represented. Only approximately 15% of the participants maintained robotic systems for template preparation or sequencing. Fi-

nally, when asked if commercial suppliers had an impact on services provided by the facilities, almost 80% of the laboratories responded affirmatively. Approximately 38% had dropped

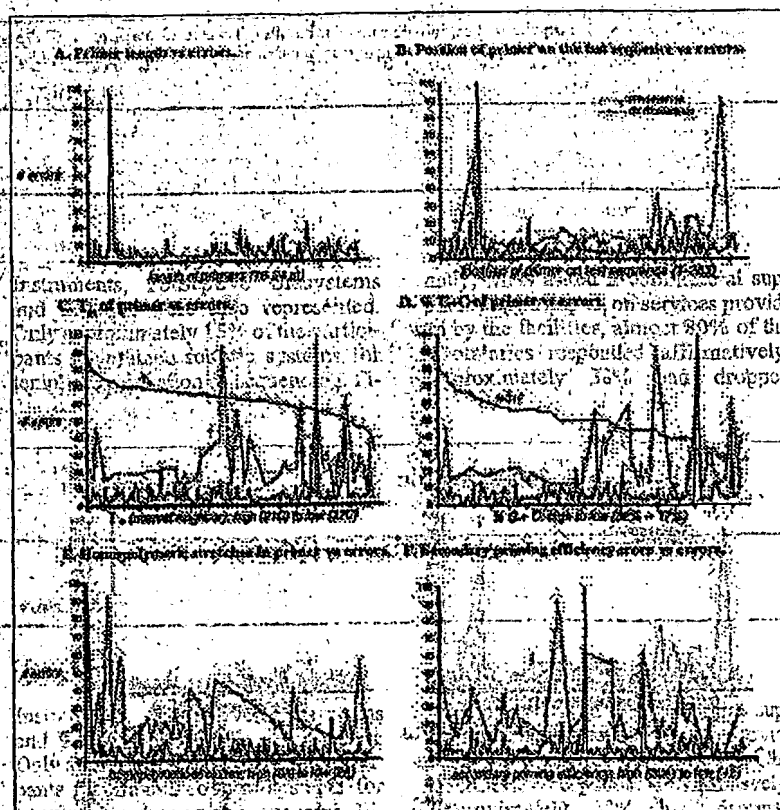


Figure 2: Primer characteristics vs. sequencing performance. The performance of sequencing primers, sorted according to various physico-chemical characteristics, was examined. The number of errors over the full-length read of each primer, determined as described in the Materials and Methods, was plotted against the results of each primer sorted according to the relevant parameter. (A) Primer length vs. errors. The primers were sorted according to their lengths in ascending order, and secondarily, according to their positions on the test sequence. The number of errors over the full-length read is plotted. Note that since all of the primers tested with the old rhodamine reagents were controls, and therefore 18-nt long, the data for old rhodamines are not included in this analysis. The X-axis distributes the primers from 16-24 nt in length; the y-axis indicates the number of errors over the full-length read of that primer. (B) Position of the primer on the test sequence. The primers were sorted according to the position of their 5' nt on the test sequence (bp 1-283 on x-axis) and the number of errors in their full-length reads were plotted (y-axis). (C) T_m of the primer. The primers were sorted in descending order by their T_m 's (91-37°C on x-axis) and the number of errors in their full-length reads were plotted (y-axis). (D) The percent G+C of the primer. The primers were sorted in descending order (82%-17% on x-axis) by their percent G+C, and the number of errors in their full-length reads were plotted (y-axis). (E) Homopolymeric stretches in the primer. The primers were sorted according to first the number of nt (8-2 on x-axis) in the longest homopolymeric stretch, and secondarily according to the number of nt in the second longest homopolymeric stretch. The number of errors over the full-length read were plotted (y-axis). (F) Secondary priming efficiency of the primers. Each primer was examined for secondary priming sites in the vector and across the test sequence using Oligo 5.0. The priming efficiency score assigned by Oligo 5.0 was determined. The primers were sorted first according to the score of the strongest secondary priming site in the vector (39-42 on x-axis) and secondarily according to the strongest secondary priming score in the test sequence. The number of errors across the full-length reads were plotted (y-axis).

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Table 2: Average Primer Performances of Submitted and Control Primers Using dRhodamine or Old Rhodamine Dye Terminator Sequencing Kits^a

	Submitted Primers dRhodamines ^b	Control Primers dRhodamines ^b	Control Repetition dRhodamines ^b	Control Primers old kit dyes ^b
5' nt trimmed ^c	3.3 (2.1-14)	4.6 (4.1-19)	4 (3.0-16)	4.5 (4.0-18)
read length in nt ^d	760 (770, 674-802)	772 (778, 683-799)	798 (803, 733-867)	752 (764, 654-803)
ambiguities (N's) ^e	2.2 (1.0-16)	1.6 (1.0-32)	4.4 (4.1-17)	8.7 (7.0-58)
errors per first 700 nt ^f	2.9 (2.0-14)	2.9 (2.0-46)	5.4 (5.3-16)	16.5 (12.3-92)

^aOld rhodamine kits refer to the original ABI Prism Dye Terminator Cycle Sequencing Kits, dRhodamine kits refer to the new ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kits containing the dichlororhodamine dye terminators.

^bThe data is shown as the average with the (median, range) in parentheses.

^cThe number of nt between the 3' end of the primer and the beginning of the trimmed sequence.

^dThe length of read in nt after trimming using Sequencher (see Materials and Methods) dRhodamine Dye Terminator Sequencing Kits^a

^eThe number of ambiguous calls (N's) in the full-length sequence read.

^fThe number of errors in the first 700 nt of the sequence.

prices and approximately 25% indicated a decreased number of synthesis requests in response to commercial suppliers. About 23% of the laboratories purchased or planned to purchase large-scale synthesis instruments to match the new competition. However, only approximately 5% had discontinued synthesis services.

Software or Manual Selection of Primers

Thirty-one laboratories described their practices for the design of sequencing and PCR primers. We were surprised that, in spite of the availability of excellent commercial primer design software programs, over 50% (17/31) of the reporting laboratories preferred to select primers manually. Of the 25 laboratories reported that selection and design of PCR primers again over 50% (14/25) tended not to use software. Of those laboratories that rely on software for primer selection, over 60% rely on various versions of the Oligo software for sequence primer design and over 70% use the Oligo software for PCR primer design.

Idealized Primer Physico-Chemical Characteristics

Participants were asked to provide their preferences for the physico-chemical characteristics of the ideal sequencing primer. We were

local characteristics of the ideal sequencing and PCR primers. This analysis provided few surprises (Table 1), in that the idealized primer characteristics of most respondents closely parallel those of some common rules of thumb for primer design. Thus, sequencing primers should be 18-24 nt in length, T_m 52°-66°C, 40%-60% G+C, with 1-2 bp 3' GC clamps (G or C nt at 3' terminus of the primer) and very little internal structure, homopolymeric sequences or potential for secondary priming. A similar set of characteristics was reported for PCR primers, although the optimal acceptable length, T_m, and percent G+C were slightly greater than for sequencing primers. However, it was unexpected that some participants accept primers of up to 40 bases, with T_m as high as 95°C for both sequencing and PCR primers. When asked to rate the relative importance of each characteristic, participants rated the existence of secondary priming sites and percent G+C as most important, with the presence of a 3' GC clamp, the potential to form primer dimers, primer length, the presence of internal structure or the presence of homopolymeric runs in order of decreasing importance for sequencing primer design. Relative importance of these characteristics was similar for PCR primers, except that the potential for formation of primer dimers was rated almost as important as

the existence of potential secondary priming sites. The 39 participating laboratories submitted 69 primer sequences ranging in length from 16-24 nt with an average of approximately 20 nt. The primers were distributed fairly evenly across the 300-bp test sequence, although some segments were obviously purposefully excluded; e.g., any primer beginning between bp 21 and 30 of the test sequence would "beat" an 8-nt poly(A) segment (not shown). Not surprisingly, most of the submitted primers conformed closely to the idealized physico-chemical characteristics for sequencing primers (Table 1). Although some deviated from the idealized characteristics, all conformed to the more broad ranges defined by participants. Thus, the participants selected primers according to their idealized criteria, even though almost half of the submitted primers were selected manually without the aid of computer software. Interestingly, some of the submitted primers had very high or low T_m (91° or 48°C) or percent G+C (82% or 29%), indicating that the participants do in fact permit a significant variation in these characteristics. Moreover, some primers exhibited relatively high internal structure, closely to the idealized physico-chemical characteristics for sequencing primers (Table 1). Although some deviated from the idealized characteristics, all conformed to the more broad ranges defined by participants. Thus, the participants selected primers according to their idealized criteria, even though almost half of the submitted primers were selected manually without the aid of computer software. Interestingly, some of the submitted primers had very high or low T_m (91° or 48°C) or percent G+C (82% or 29%), indicating that the participants do in fact permit a significant variation in these characteristics. Moreover, some primers exhibited relatively high internal structure, closely to the idealized physico-chemical characteristics for sequencing primers (Table 1).

secondary priming efficiency numbers (a score calculated by Oligo 5.0; whereby a priming efficiency of 160 can prime in sequencing reactions) even within the test sequence (Table 1).

Sequencing Performance of the Primers

The results of the sequence analysis as described in the Materials and Methods were unexpected (Table 2); i.e., we expected a wide range in the performance of the submitted and control primers. Instead, all of the submitted primers functioned extremely well, the poorest performer still yielding <2% errors over a >700-nt sequence. The average number of errors and ambiguities over the 700-nt window was 2.9, and the average read length was 760 nt. Similarly, almost all of the control primers functioned very well, with an average of 2.9 errors or ambiguities over the 700-nt window and an average read length of 772 bases. Only one primer (No. 8) failed to give any reasonable sequence and even this oligonucleotide performed adequately on the repeat, i.e., 13 errors and ambiguities over a 700-nt read. The average read lengths of the sequences from the submitted and control primers were 760 and 772, with ranges of 674–802 and 683–799 bases, respectively. The average number of nucleotides trimmed from the 5' ends of these sequences was 3.3 and 4.6 nt, respectively. Many sequences began on the first nucleotide after the primers, but up to 14 or 19 nt were trimmed from the 5' ends of the submitted and control primers. As expected from previous studies (12,14,18), errors and ambiguities were clustered in the first and last 100-bp windows of the sequences (data not shown). Approximately 30% of the control primers were used in repeat reactions with very similar results (Table 2 and data not shown).

The control primers used in sequence reactions using the old rhodamine dye terminator reagents gave slightly poorer overall results. The read lengths of these reactions did not differ greatly from the read lengths of the dRhodamine reactions (average 752 nt vs. 772 bases, Table 2). However, there were more N's (average 8.7 vs. 1.6) and

errors and ambiguities (average 16.5 vs. 2.9) in the data generated with old rhodamine dye terminators than in the dRhodamine-generated data (Table 2).

Poor Primers Yield Good Sequence Results

The uniform high-quality sequence data from the control primers using the dRhodamine reagents was not expected. Only primer No. 8 failed catastrophically (i.e., gave no valid sequence data), and primer No. 9 yielded >5% errors over a 700-nt window. However, both of these primers provided better data when used in a repeat sequencing reaction (Table 3). Moreover, there was no obvious relationship between the number of errors in a sequence and the length, position, Tm, percent G+C, secondary priming potential, length of homopolymeric stretches (Figure 2, panels A–F) or the potential for forming primer dimers (data not shown) of the primers. On the contrary, many primers exhibiting suboptimal physico-chemical characteristics yielded high-quality sequence. For example, although the one primer No. 8 that failed catastrophically has an 8-nt poly(dA) stretch near its 3' terminus, other primers, e.g., Nos. 7 and 10, contain a similar sequence, but still generated very respectable data (Table 3). Primer No. 9, which also contains the same poly(dA) stretch, also yielded relatively poor data (46 errors in 700 bases), although the error rate was <3% over the first 500-nt window (data not shown). Examination of the sequences of these primers suggests that homopolymeric stretches of dA's may have a negative impact on sequence quality, but that these stretches must be quite long, and near to the 3' terminus to exert their effects. Other primers with extensive homopolymeric stretches yielded good results (Table 3). Finally, when the length of the longest homopolymeric stretch in each of the primers was plotted against the number of errors in the sequence reaction (Figure 2B), no general trend was observed when reactions were performed with dRhodamine reagents. Together, these observations suggest that under optimal conditions using dRhodamine reagents, long homopolymeric stretches do not necessarily negatively

number of errors over a 700-nt sequencing window was 2.9, 3.1 and 3.3 for all submitted primers, for submitted primers selected using software and for the control primers, respectively. Moreover, no clear trends were observed when comparing the physico-chemical characteristics of the software-designed primers to the manually designed or the control primers (data not shown).

Old Rhodamines vs. dRhodamines

The old rhodamine dye reactions were more strongly impacted by primer physico-chemical characteristics than the dRhodamine dye reactions. As described above, the old rhodamine dye reactions were consistently poorer than the dRhodamine reactions (Table 2). Moreover, the former reactions seemed more sensitive to primer characteristics. Thus, reactions with higher error rates tended to be those with lower T_m , lower percent G+C and longer homopolymeric stretches (Figure 2, panels C-E). As with the dRhodamines, the old rhodamine reactions exhibited no discernable trend in relation to secondary priming potential (Figure 2F), or primer secondary structure (data not shown).

DISCUSSION

In this study, we examined the perceptions and strategies of participant core facility personnel for the design and selection of DNA sequencing and PCR primers. The results may reveal some unexpected misconceptions in the requirements for good sequencing primer design. Thus, most laboratories espouse primer design dogma suggesting that a sequencing primer should be 18-24 nt in length with a T_m of 52°-66°C, 40%-60% G+C, 1-2 bp 3' GC clamps and little internal structure, homopolymeric sequence or potential for secondary priming. The percent G+C and secondary priming potential were rated as the most important characteristics, but T_m , GC clamps, primer secondary and tertiary structure, length and the presence of homopolymeric runs were also thought to be relevant. Moreover, previous studies have shown that secondary structure of the template in the primer target regions can effect

priming efficiency (7,8).

With regard to primer design, we were surprised to find that the majority of investigators design most of their sequencing primers manually. Thus, in spite of the perceived importance of primer characteristics and the widespread availability of primer design software, approximately 55% of the participants in this study elected to design primers manually. We believe that this observation is the result of a combination of the facts that primer design software is not yet sufficiently "user-friendly" and that the primer physico-chemical characteristics are not as important as commonly believed. It would seem that the authors of primer design software could take note of this result and streamline and enhance their products so that they are more convenient, beneficial and practical to use. Clearly, however, there are many circumstances (e.g., templates with GC-rich regions or with unusual sequences or structures, genomic sequencing or the sequencing of very large templates with many repeat regions) in which it would be advisable to use appropriate primer design software to automate and expedite primer design and synthesis.

The results of the empirical sequencing analysis were surprising in that nearly all of the primers yielded data of extremely high quality. Excellent data was obtained in spite of widely varying primer T_m and percent G+C, the presence of nearly 50% of the primer as homopolymer and the presence of very strong secondary priming sites. Characteristics considered by most respondents to be the critical for sequencing primers. This study confirms earlier observations (3) that there is a broad tolerance in these and other characteristics of sequencing primers. Other factors, such as template purity or quality or technical expertise, possibly play greater roles. In this study, the plasmid template was selected for absence of sequence extension obstacles and purified by double banding in CsCl-ethidium bromide isopycnic density gradients. Therefore, this template was extremely pure and optimal for sequencing. The primers were similarly highly purified. The reactions were performed in a single high-throughput sequencing facility under tightly

Exhibit G

▷

In re Deuel
C.A.Fed.,1995.

United States Court of Appeals, Federal Circuit.
In re Thomas F. DEUEL, Yue-Sheng Li, Ned R.
Siegel and Peter G. Milner.
No. 94-1202.

March 28, 1995.

Inventors applied for patent for deoxyribonucleic acid (DNA) and complementary DNA (cDNA) molecules encoding proteins that stimulated cell division. After patent examiner rejected claims as unpatentable on grounds of obviousness and the Patent and Trademark Office Board of Patent Appeals and Interferences affirmed, inventors appealed. The Court of Appeals, Lourie, Circuit Judge, held that: (1) combination of prior art reference teaching method of gene cloning, together with reference disclosing partial amino acid sequence for a protein that stimulated cell division, did not render claims prima facie obvious; (2) conceived method of preparing some unidentified DNA does not define it with precision necessary to render it obvious over protein it encodes; and (3) patent claims generically encompassing all DNA sequences encoding human and bovine proteins to stimulate cell division were not invalid as obvious.

Reversed.

West Headnotes

[1] Patents 291 🔑314(5)

291 Patents

291XII Infringement

291XII(C) Suits in Equity

291k314 Hearing

291k314(5) k. Questions of Law or Fact.

Most Cited Cases

Patents 291 🔑324.55(1)

291 Patents

291XII Infringement

291XII(C) Suits in Equity

291k324 Appeal

291k324.55 Questions of Fact, Verdicts,
and Findings

291k324.55(1) k. In General. Most

Cited Cases

Obviousness is question of law, which Court of Appeals reviews de novo, though factual findings underlying obviousness determination are reviewed for clear error. 35 U.S.C.A. § 103.

[2] Patents 291 🔑32

291 Patents

291II Patentability

291II(A) Invention; Obviousness

291k31 Evidence of Invention

291k32 k. Presumptions and Burden of Proof. Most Cited Cases

Patent examiner bears burden of establishing prima facie case of obviousness; only if this burden is met does burden of coming forward with rebuttal argument or evidence shift to applicant. 35 U.S.C.A. § 103.

[3] Patents 291 🔑36(1)

291 Patents

291II Patentability

291II(A) Invention; Obviousness

291k36 Weight and Sufficiency

291k36(1) k. In General. Most Cited Cases

Patents 291 🔑324.55(4)

291 Patents

291XII Infringement

291XII(C) Suits in Equity

291k324 Appeal

291k324.55 Questions of Fact, Verdicts,
and Findings

291k324.55(3) Issues of Validity

291k324.55(4) k. Novelty,
Invention, Anticipation, and Obviousness. Most Cited Cases

When references cited by patent examiner fail to establish prima facie case of obviousness, rejection on ground of obviousness is improper and will be overturned. 35 U.S.C.A. § 103.

[4] Patents 291 🔑16.3

291 Patents

291II Patentability

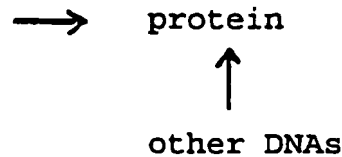
291II(A) Invention; Obviousness

relationship between codons and amino acids. The reverse is not true, however, due to the degeneracy of the code. Many other DNAs may code for a particular protein. The functional relationships

genomic



between DNA, mRNA, cDNA, and a protein may conveniently be expressed as follows:



nucleotide sequence, Deuel then predicted the complete amino acid sequence of bovine uterine HBGF disclosed in Deuel's application.

Collections ("libraries") of DNA and cDNA molecules derived from various species may be constructed in the laboratory or obtained from commercial sources. Complementary DNA libraries contain a mixture of cDNA clones reverse-transcribed from the mRNAs found in a specific tissue source. Complementary DNA libraries are tissue-specific because proteins and their corresponding mRNAs are only made ("expressed") in specific tissues, depending upon the protein. Genomic DNA ("gDNA") libraries, by contrast, theoretically contain all of a species' chromosomal DNA. The molecules present in cDNA and DNA libraries may be of unknown function and chemical structure, and *1555 the proteins which they encode may be unknown. However, one may attempt to retrieve molecules of interest from cDNA or gDNA libraries by screening such libraries with a gene probe, which is a synthetic radiolabelled nucleic acid sequence designed to bond ("hybridize") with a target complementary base sequence. Such "gene cloning" techniques thus exploit the fact that the bases in DNA always hybridize in complementary pairs: adenine bonds with thymine and guanine bonds with cytosine. A gene probe for potentially isolating DNA or cDNA encoding a protein may be designed once the protein's amino acid sequence, or a portion thereof, is known.

As disclosed in Deuel's patent application, Deuel isolated and purified HBGF from bovine uterine tissue, found that it exhibited mitogenic activity, and determined the first 25 amino acids of the protein's N-terminal sequence. ^{FN2} Deuel then isolated a cDNA molecule encoding bovine uterine HBGF by screening a bovine uterine cDNA library with an oligonucleotide probe designed using the experimentally determined N-terminal sequence of the HBGF. Deuel purified and sequenced the cDNA molecule, which was found to consist of a sequence of 1196 nucleotide base pairs. From the cDNA's

FN2. Deuel determined that the N-terminal sequence of bovine uterus HBGF is Gly-Lys-Lys-Glu-Lys-Pro-Glu-Lys-Lys-Val-Lys-Lys-Ser-Asp-Cys-Gly-Glu-Trp-Gln-Trp-Ser-Val-Cys-Val-Pro.

Deuel also isolated a cDNA molecule encoding human placental HBGF by screening a human placental cDNA library using the isolated bovine uterine cDNA clone as a probe. Deuel purified and sequenced the human placental cDNA clone, which was found to consist of a sequence of 961 nucleotide base pairs. From the nucleotide sequence of the cDNA molecule encoding human placental HBGF, Deuel predicted the complete amino acid sequence of human placental HBGF disclosed in Deuel's application. The predicted human placental and bovine uterine HBGFs each have 168 amino acids and calculated molecular weights of 18.9 kD. Of the 168 amino acids present in the two HBGFs discovered by Deuel, 163 are identical. Deuel's application does not describe the chemical structure of, or state how to isolate and purify, any DNA or cDNA molecule except the disclosed human placental and bovine uterine cDNAs, which are the subject of claims 5 and 7.

Claims 4-7 on appeal are all independent claims and read, in relevant part, as follows:

4. A purified and isolated DNA sequence consisting of a sequence encoding human heparin binding growth factor of 168 amino acids having the following amino acid sequence: Met Gln Ala ... [remainder of 168 amino acid sequence].
5. The purified and isolated cDNA of human heparin-binding growth factor having the following nucleotide sequence: GTCAAAGGCA ... [remainder of 961 nucleotide sequence].
6. A purified and isolated DNA sequence consisting of a sequence encoding bovine heparin binding

291III Patentability

291III(A) Invention; Obviousness

291k26 Combination

291k26(1) k. In General. Most Cited

Cases

Conceived method of preparing some unidentified deoxyribonucleic acid (DNA) does not define it with precision necessary to render it obvious over protein it encodes. 35 U.S.C.A. § 103.

[11] Patents 291 26(1)

291 Patents

291III Patentability

291III(A) Invention; Obviousness

291k26 Combination

291k26(1) k. In General. Most Cited

Cases

Patent claims generically encompassing all deoxyribonucleic acid (DNA) sequences encoding human and bovine protein to stimulate cell division were not invalid as obvious, where prior art disclosed only partial amino acid sequence for the protein. 35 U.S.C.A. § 103.

G. Harley Blosser, Senniger, Powers, Leavitt & Roedel, of St. Louis, MO, argued for appellants. With him on the brief was Donald G. Leavitt.

Donald S. Chisum, Morrison & Foerster, Seattle, WA, argued for amicus curiae, The Biotechnology Industry Ass'n and The Bay Area Bioscience Center. With him on the brief were Debra A. Shetka, Morrison & Forester, Palo Alto, CA and Robert P. Blackburn, Emeryville, CA.

Teddy S. Gron, Acting Associate Sol., Arlington, VA, argued for appellee. With him on the brief was Albin F. Drost, Acting Sol. Nancy J. Linck, Office of the Sol., Arlington, VA, represented appellee.

Before ARCHER, Chief Judge, NIES and LOURIE, Circuit Judges.

LOURIE, Circuit Judge.

Thomas F. Deuel, Yue-Sheng Li, Ned R. Siegel, and Peter G. Milner (collectively "Deuel") appeal from the November 30, 1993 decision of the U.S. Patent and Trademark Office Board of Patent Appeals and Interferences affirming the examiner's final rejection of claims 4-7 of application Serial No. 07/542,232, entitled "Heparin-Binding *1554 Growth Factor," as unpatentable on the ground of obviousness under 35 U.S.C. § 103 (1988). Ex parte Deuel, 33 USPQ2d 1445 (Bd.Pat.App.Int.1993). Because the Board erred in concluding that Deuel's claims 5 and 7 directed to specific cDNA molecules would have

been obvious in light of the applied references, and no other basis exists in the record to support the rejection with respect to claims 4 and 6 generically covering all possible DNA molecules coding for the disclosed proteins, we reverse.

BACKGROUND

The claimed invention relates to isolated and purified DNA and cDNA molecules encoding heparin-binding growth factors ("HBGFs").^{FN1} HBGFs are proteins that stimulate mitogenic activity (cell division) and thus facilitate the repair or replacement of damaged or diseased tissue. DNA (deoxyribonucleic acid) is a generic term which encompasses an enormous number of complex macromolecules made up of nucleotide units. DNAs consist of four different nucleotides containing the nitrogenous bases adenine, guanine, cytosine, and thymine. A sequential grouping of three such nucleotides (a "codon") codes for one amino acid. A DNA's sequence of codons thus determines the sequence of amino acids assembled during protein synthesis. Since there are 64 possible codons, but only 20 natural amino acids, most amino acids are coded for by more than one codon. This is referred to as the "redundancy" or "degeneracy" of the genetic code.

FN1. For a more extensive discussion of recombinant DNA technology, see In re O'Farrell, 853 F.2d 894, 895-99, 7 USPQ2d 1673, 1674-77 (Fed.Cir.1988); Amgen Inc. v. Chugai Pharmaceutical Co., 927 F.2d 1200, 18 USPQ2d 1016 (Fed.Cir.), cert. denied, 502 U.S. 856, 112 S.Ct. 169, 116 L.Ed.2d 132 (1991).

DNA functions as a blueprint of an organism's genetic information. It is the major component of genes, which are located on chromosomes in the cell nucleus. Only a small part of chromosomal DNA encodes functional proteins.

Messenger ribonucleic acid ("mRNA") is a similar molecule that is made or transcribed from DNA as part of the process of protein synthesis. Complementary DNA ("cDNA") is a complementary copy ("clone") of mRNA, made in the laboratory by reverse transcription of mRNA. Like mRNA, cDNA contains only the protein-encoding regions of DNA. Thus, once a cDNA's nucleotide sequence is known, the amino acid sequence of the protein for which it codes may be predicted using the genetic code

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Table 3. Primers with Physico-Chemical Characteristics that Were Not Expected to Provide Good Sequencing Results

Primer# Sequence			Errors				"old" Rhodamine's	
			dRhodamines					
			per 700 nt	total read	per 700 nt	total read	per 700 nt	total read
Homopolymeric stretches								
86	TTTACCCCTTCTTCAAA	AA/40	5	6/784	4	5/203	4	58/585
87	CTCTCTTCTTCAAA	TA/40	4	9/756	5	16/430	10	13/746
88	CTCTCTTCTTCAAA	GA/30	23/200		23	17/613		62/558
89	TTTCAAAATTAATTA	CA/28	4	45/483	7	15/443	37	63/714
90	TTTCAAAATTAATTA	GA/28	1	9/795	3	11/780	11	22/721
976	ATTTTCAAAATTAATTA	GA/30	2	7/787	23	NA	NA	NA
977	ATTTTCAAAATTAATTA	CA/44	0	3/773	NA	NA	NA	NA
Multiple homopolymeric stretches								
910a	CTCTCTTCTTCAAA	AT/40	3	5/773	NA	NA	NA	NA
984	CTCTCTTCTTCAAA	AT/40	1	9/774	10	13/746	10	23/546
980	CTCTCTTCTTCAAA	TA/40	1	11/771	5	21/467	15	13/733
979	CTCTCTTCTTCAAA	TA/40	2	1/760	7	9/775	12	13/732
973	CTCTCTTCTTCAAA	TA/40	1	3/763	NA	NA	16	92/780
Secondary priming sites								
983	CTCTCTTCTTCAAA	AT/40	0	9/771	5	16/430	4	13/746
985	CTCTCTTCTTCAAA	AT/40	1	45/483	7	15/443	37	63/714
976	CTCTCTTCTTCAAA	AT/40	2	7/787	23	NA	NA	NA
977	CTCTCTTCTTCAAA	AT/40	4	11/784	NA	NA	NA	NA
910a	CTCTCTTCTTCAAA	AT/40	1	5/773	NA	NA	NA	NA

¹Errors and ambiguities per 700 nt read, or per total read length. For the dRhodamines, both the initial results and the results of the repeat analysis are shown.

²Length of the longest and second longest homopolymeric stretches in the primer.

³X/Y where X is the priming efficiency number generated by Oligo version 5.0, for potential secondary priming in the vector, and Y is the value for a perfect match with that primer. In general, the higher the score, the higher the stability and quality of the priming at that site. A score of >200 will often give false priming results according to the Oligo 5.0 manual. nd, not determined.

effect sequence results.

T_m and percent G+C also showed less impact than expected on the dRhodamine reactions (Figure 2, C and D, and Table 3). Thus, primers with low T_m and percent G+C, e.g., 37°C/28% and 44°C/28% for primer Nos. 72 and 39, and others with high T_m /percent G+C, e.g., 75°C/72% and 83°C/78% for control primer Nos. 80 and 84 and 91°C/82% for submitted primer No.

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4321b, each provided sequence at well under 1% error rate (Table 3). Secondary priming potential also exhibited less of an obstacle for dRhodamine sequencing than was anticipated. Primers with very high secondary priming scores at sites within the template provided excellent sequence data. Control primer Nos. 82 and 83, and submitted primer Nos. 10Ab and 49b, had high secondary priming scores, and in each

case the homology encompassed the 3' end of the primer (Table 3). Nonetheless, these primers yielded very high-quality results.

Finally, we compared the sequencing results generated with primers selected by software to the results of manually selected primers and to the arbitrarily selected control primers. There was no observable difference in the results obtained; i.e., the average

length of the longest and second longest homopolymeric stretches in the primer, as determined by Oligo version 5.0, for potential secondary priming in the vector, and Y is the value for a perfect match with that primer. In general, the higher the score, the higher the stability and quality of the priming at that site. A score of >200 will often give false priming results according to the Oligo 5.0 manual.

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controlled conditions. Different results may be obtained using less carefully purified DNA templates with unusual sequences or structures or in less rigorously controlled sequencing operations.

Finally, the data generated using old rhodamine reagents was, in general, poorer in quality than that generated using the dRhodamine reagents. Moreover, the expected trends in comparing sequence performance and primer characteristics were more prominently observed using the old rhodamine reagents. We believe it possible, if not likely, that the greater uniformity in peak height generated by the newer sequencing reagents (19) is at least partially responsible for this difference. Thus, background peaks are less likely to interfere with base calling, lessening the overall effects of potential problems like secondary priming and stutter.

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ACKNOWLEDGMENTS

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291k16.3 k. Natural or Scientific Phenomena or Principles. Most Cited Cases

Patents 291  16.25

291 Patents

291II Patentability

291II(A) Invention; Obviousness

291k16.25 k. Chemical Compounds. Most Cited Cases

Combination of prior art reference teaching method of gene cloning, together with reference disclosing partial amino acid sequence for a protein that stimulated cell division, did not render deoxyribonucleic acid (DNA) and complementary DNA (cDNA) molecules encoding protein prima facie obvious; prior art did not disclose any relevant cDNA molecules, let alone close relatives of specific, structurally defined cDNA molecules of patent claims. 35 U.S.C.A. § 103.

[5] Patents 291  16.3

291 Patents

291II Patentability

291II(A) Invention; Obviousness

291k16.3 k. Natural or Scientific Phenomena or Principles. Most Cited Cases

Patents 291  16.25

291 Patents

291II Patentability

291II(A) Invention; Obviousness

291k16.25 k. Chemical Compounds. Most Cited Cases

Prior art disclosure of amino acid sequence of protein does not necessarily render particular deoxyribonucleic acid (DNA) molecules encoding protein obvious because redundancy of genetic code permits one to hypothesizing enormous number of DNA sequences coding for the protein; no particular one of these DNAs can be obvious unless there is something in prior art to lead to particular DNA and indicate that it should be prepared. 35 U.S.C.A. § 103.

[6] Patents 291  16.25

291 Patents

291II Patentability

291II(A) Invention; Obviousness

291k16.25 k. Chemical Compounds. Most Cited Cases

Existence of general method of isolating complementary deoxyribonucleic acid (cDNA) or DNA molecules is essentially irrelevant to question of whether specific molecules themselves would have been obvious, in absence of other prior art that suggests claimed DNAs. 35 U.S.C.A. § 103.

[7] Patents 291  26(1)

291 Patents

291II Patentability

291II(A) Invention; Obviousness

291k26 Combination

291k26(1) k. In General. Most Cited Cases

Where there is prior art that suggests claimed compound, existence, or lack thereof, of enabling process for making that compound is factor in any patentability determination; there must, however, still be prior art that suggests claimed compound in order for prima facie case of obviousness to be made out. 35 U.S.C.A. § 103.

[8] Patents 291  16(1)

291 Patents

291II Patentability

291II(A) Invention; Obviousness

291k16 Invention and Obviousness in General

291k16(1) k. In General. Most Cited Cases

General incentive does not make obvious particular result, nor does existence of techniques by which to make those efforts to be carried out. 35 U.S.C.A. § 103.

[9] Patents 291  26(1)

291 Patents

291II Patentability

291II(A) Invention; Obviousness

291k26 Combination

291k26(1) k. In General. Most Cited Cases

Fact that one can conceive general process in advance for preparing undefined compound does not mean that claimed specific compound was precisely envisioned and therefore obvious. 35 U.S.C.A. § 103.

[10] Patents 291  26(1)

291 Patents

growth factor of 168 amino acids having the following amino acid sequence: Met Gln Thr ... [remainder of 168 amino acid sequence].

7. The purified and isolated cDNA of bovine heparin-binding growth factor having the following nucleotide sequence: GAGTGGAGAG ... [remainder of 1196 nucleotide sequence].

Claims 4 and 6 generically encompass *all* isolated/purified DNA sequences (natural and synthetic) encoding human and bovine HBGFs, despite the fact that Deuel's application does not describe the chemical structure of, or tell how to obtain, any DNA or cDNA except the two disclosed cDNA molecules. Because of the redundancy of the genetic code, claims 4 and 6 each encompass an enormous number of DNA molecules, including the isolated/purified chromosomal DNAs encoding the human and bovine proteins. Claims 5 and 7, on the other hand, are directed to the specifically disclosed cDNA molecules encoding human and bovine HBGFs, respectively.

During prosecution, the examiner rejected claims 4-7 under 35 U.S.C. § 103 as unpatentable over the combined teachings of Bohlen*1556 ^{FN3} and Maniatis.^{FN4} The Bohlen reference discloses a group of protein growth factors designated as heparin-binding brain mitogens ("HBBMs") useful in treating burns and promoting the formation, maintenance, and repair of tissue, particularly neural tissue. Bohlen isolated three such HBBMs from human and bovine brain tissue. These proteins have respective molecular weights of 15 kD, 16 kD, and 18 kD. Bohlen determined the first 19 amino acids of the proteins' N-terminal sequences, which were found to be identical for human and bovine HBBMs.^{FN5} Bohlen teaches that HBBMs are brain-specific, and suggests that the proteins may be homologous between species. The reference provides no teachings concerning DNA or cDNA coding for HBBMs.

^{FN3}. European Patent Application No. 0326075, naming Peter Bohlen as inventor, published August 2, 1989.

^{FN4}. Maniatis et al., *Molecular Cloning: A Laboratory Manual*, "Screening Bacteriophage [lambda] Libraries for Specific DNA Sequences by Recombination in *Escherichia coli*," Cold Spring Harbor Laboratory, New York, 1982, pp. 353-361.

^{FN5}. Bohlen's disclosed N-terminal sequence for human and bovine HBBMs is Gly-Lys-Lys-Glu-Lys-Pro-Glu-Lys-Lys-Val-Lys-Lys-Ser-Asp-Cys-Gly-Glu-Trp-Gln. This sequence matches the first 19 amino acids of Deuel's disclosed N-terminal sequence.

Maniatis describes a method of isolating DNAs or cDNAs by screening a DNA or cDNA library with a gene probe. The reference outlines a general technique for cloning a gene; it does not describe how to isolate a particular DNA or cDNA molecule. Maniatis does not discuss certain steps necessary to isolate a target cDNA, *e.g.*, selecting a tissue-specific cDNA library containing a target cDNA and designing an oligonucleotide probe that will hybridize with the target cDNA.

The examiner asserted that, given Bohlen's disclosure of a heparin-binding protein and its N-terminal sequence and Maniatis's gene cloning method, it would have been *prima facie* obvious to one of ordinary skill in the art at the time of the invention to clone a gene for HBGF.^{FN6} According to the examiner, Bohlen's published N-terminal sequence would have motivated a person of ordinary skill in the art to clone such a gene because cloning the gene would allow recombinant production of HBGF, a useful protein. The examiner reasoned that a person of ordinary skill in the art could have designed a gene probe based on Bohlen's disclosed N-terminal sequence, then screened a DNA library in accordance with Maniatis's gene cloning method to isolate a gene encoding an HBGF. The examiner did not distinguish between claims 4 and 6 generically directed to all DNA sequences encoding human and bovine HBGFs and claims 5 and 7 reciting particular cDNAs.

^{FN6}. The examiner and the Board apparently used the term "gene" to refer both to natural (chromosomal) DNA and synthetic cDNA. We will use the several terms as appropriate.

In reply, Deuel argued, *inter alia*, that Bohlen teaches away from the claimed cDNA molecules because Bohlen suggests that HBBMs are brain-specific and, thus, a person of ordinary skill in the art would not have tried to isolate corresponding cDNA clones from human placental and bovine uterine cDNA libraries. The examiner made the rejection final,

however, asserting that [t]he starting materials are not relevant in this case, because it was well known in the art at the time the invention was made that proteins, especially the general class of heparin binding proteins, are highly homologous between species and tissue type. It would have been entirely obvious to attempt to isolate a known protein from different tissue types and even different species.

No prior art was cited to support the proposition that it would have been obvious to screen human placental and bovine uterine cDNA libraries for the claimed cDNA clones. Presumably, the examiner was relying on Bohlen's suggestion that HBBMs may be homologous between species, although the examiner did not explain how homology between species suggests homology between tissue types.

The Board affirmed the examiner's final rejection. In its opening remarks, the Board noted that it is "constantly advised by the *1557 patent examiners, who are highly skilled in this art, that cloning procedures are routine in the art." According to the Board, "the examiners urge that when the sequence of a protein is placed into the public domain, the gene is also placed into the public domain because of the routine nature of cloning techniques." Addressing the rejection at issue, the Board determined that Bohlen's disclosure of the existence and isolation of HBBM, a functional protein, would also advise a person of ordinary skill in the art that a gene exists encoding HBBM. The Board found that a person of ordinary skill in the art would have been motivated to isolate such a gene because the protein has useful mitogenic properties, and isolating the gene for HBBM would permit large quantities of the protein to be produced for study and possible commercial use. Like the examiner, the Board asserted, without explanation, that HBBMs are the same as HBGFs and that the genes encoding these proteins are identical. The Board concluded that "the Bohlen reference would have suggested to those of ordinary skill in this art that they should make the gene, and the Maniatis reference would have taught a technique for 'making' the gene with a reasonable expectation of success." Responding to Deuel's argument that the claimed cDNA clones were isolated from human placental and bovine uterine cDNA libraries, whereas the combined teachings of Bohlen and Maniatis would only have suggested screening a brain tissue cDNA library, the Board stated that "the claims before us are directed to the product and not the method of isolation. Appellants have not shown that the claimed DNA was not present in and could not

have been readily isolated from the brain tissue utilized by Bohlen." Deuel now appeals.^{FN7}

FN7. Deuel is supported in its appeal by an *amicus curiae* brief submitted by the Biotechnology Industry Organization and the Bay Area Science Center. Amici urge that, contrary to controlling precedent, the PTO has unlawfully adopted a *per se* rule that a gene is *prima facie* obvious when at least part of the amino acid sequence of the protein encoded by the gene is known in the prior art.

DISCUSSION

[1][2][3] Obviousness is a question of law, which we review *de novo*, though factual findings underlying the Board's obviousness determination are reviewed for clear error. *In re Vaeck*, 947 F.2d 488, 493, 20 USPQ2d 1438, 1442 (Fed.Cir.1991); *In re Woodruff*, 919 F.2d 1575, 1577, 16 USPQ2d 1934, 1935 (Fed.Cir.1990). The examiner bears the burden of establishing a *prima facie* case of obviousness. *In re Rijckaert*, 9 F.3d 1531, 1532, 28 USPQ2d 1955, 1956 (Fed.Cir.1993); *In re Oetiker*, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed.Cir.1992). Only if this burden is met does the burden of coming forward with rebuttal argument or evidence shift to the applicant. *Rijckaert*, 9 F.3d at 1532, 28 USPQ2d at 1956. When the references cited by the examiner fail to establish a *prima facie* case of obviousness, the rejection is improper and will be overturned. *In re Fine*, 837 F.2d 1071, 1074, 5 USPQ2d 1596, 1598 (Fed.Cir.1988).

[4] On appeal, Deuel challenges the Board's determination that the applied references establish a *prima facie* case of obviousness. In response, the PTO maintains that the claimed invention would have been *prima facie* obvious over the combined teachings of Bohlen and Maniatis. Thus, the appeal raises the important question whether the combination of a prior art reference teaching a method of gene cloning, together with a reference disclosing a partial amino acid sequence of a protein, may render DNA and cDNA molecules encoding the protein *prima facie* obvious under § 103.

Deuel argues that the PTO failed to follow the proper legal standard in determining that the claimed cDNA molecules would have been *prima facie* obvious despite the lack of structurally similar compounds in the prior art. Deuel argues that the PTO has not

cited a reference teaching cDNA molecules, but instead has improperly rejected the claims based on the alleged obviousness of a method of making the molecules. We agree.

Because Deuel claims new chemical entities in structural terms, a *prima facie* case of unpatentability requires that the teachings of the prior art suggest *the claimed compounds* to a person of ordinary skill in the art. *1558 Normally a *prima facie* case of obviousness is based upon structural similarity, *i.e.*, an established structural relationship between a prior art compound and the claimed compound. Structural relationships may provide the requisite motivation or suggestion to modify known compounds to obtain new compounds. For example, a prior art compound may suggest its homologs because homologs often have similar properties and therefore chemists of ordinary skill would ordinarily contemplate making them to try to obtain compounds with improved properties. Similarly, a known compound may suggest its analogs or isomers, either geometric isomers (*cis v. trans*) or position isomers (*e.g.*, *ortho v. para*).

In all of these cases, however, the prior art teaches a specific, structurally-definable compound and the question becomes whether the prior art would have suggested making the specific molecular modifications necessary to achieve the claimed invention. See In re Jones, 958 F.2d 347, 351, 21 USPQ2d 1941, 1944 (Fed.Cir.1992); In re Dillon, 919 F.2d 688, 692, 16 USPQ2d 1897, 1901 (Fed.Cir.1990) (en banc) (“structural similarity between claimed and prior art subject matter, ... where the prior art gives reason or motivation to make the claimed compositions, creates a *prima facie* case of obviousness”), *cert. denied*, 500 U.S. 904, 111 S.Ct. 1682, 114 L.Ed.2d 77 (1991); In re Grabiak, 769 F.2d 729, 731-32, 226 USPQ 870, 872 (Fed.Cir.1985) (“[I]n the case before us there must be adequate support in the prior art for the [prior art] ester/[claimed] thioester change in structure, in order to complete the PTO's *prima facie* case and shift the burden of going forward to the applicant.”); In re Lahu, 747 F.2d 703, 705, 223 USPQ 1257, 1258 (Fed.Cir.1984) (“The prior art must provide one of ordinary skill in the art the motivation to make the proposed molecular modifications needed to arrive at the claimed compound.”).

Here, the prior art does not disclose any relevant cDNA molecules, let alone close relatives of the specific, structurally-defined cDNA molecules of claims 5 and 7 that might render them obvious.

Maniatis suggests an allegedly obvious process for trying to isolate cDNA molecules, but that, as we will indicate below, does not fill the gap regarding the subject matter of claims 5 and 7. Further, while the general idea of the claimed molecules, their function, and their general chemical nature may have been obvious from Bohlen's teachings, and the knowledge that some gene existed may have been clear, the precise cDNA molecules of claims 5 and 7 would not have been obvious over the Bohlen reference because Bohlen teaches proteins, not the claimed or closely related cDNA molecules. The redundancy of the genetic code precluded contemplation of or focus on the specific cDNA molecules of claims 5 and 7. Thus, one could not have conceived the subject matter of claims 5 and 7 based on the teachings in the cited prior art because, until the claimed molecules were actually isolated and purified, it would have been highly unlikely for one of ordinary skill in the art to contemplate what was ultimately obtained. What cannot be contemplated or conceived cannot be obvious.

The PTO's theory that one might have been motivated to try to do what Deuel in fact accomplished amounts to speculation and an impermissible hindsight reconstruction of the claimed invention. It also ignores the fact that claims 5 and 7 are limited to specific compounds, and any motivation that existed was a general one, to try to obtain a gene that was yet undefined and may have constituted many forms. A general motivation to search for some gene that exists does not necessarily make obvious a specifically-defined gene that is subsequently obtained as a result of that search. More is needed and it is not found here.

[5] The genetic code relationship between proteins and nucleic acids does not overcome the deficiencies of the cited references. A prior art disclosure of the amino acid sequence of a protein does not necessarily render particular DNA molecules encoding the protein obvious because the redundancy of the genetic code permits one to hypothesize an enormous number of DNA sequences coding for the protein. No particular one of these DNAs can be obvious unless there is something in the prior art to lead to the particular DNA and indicate that it should be *1559 prepared. We recently held in In re Baird, 16 F.3d 380, 29 USPQ2d 1550 (Fed.Cir.1994), that a broad genus does not necessarily render obvious each compound within its scope. Similarly, knowledge of a protein does not give one a conception of a particular DNA encoding it. Thus, *a fortiori*, Bohlen's disclosure of the N-terminal portion of a

protein, which the PTO urges is the same as HBGF, would not have suggested the particular cDNA molecules defined by claims 5 and 7. This is so even though one skilled in the art knew that some DNA, albeit not in purified and isolated form, did exist. The compounds of claims 5 and 7 are specific compounds not suggested by the prior art. A different result might pertain, however, if there were prior art, e.g., a protein of sufficiently small size and simplicity, so that lacking redundancy, each possible DNA would be obvious over the protein. See *In re Petering*, 301 F.2d 676 (CCPA 1962) (prior art reference disclosing limited genus of 20 compounds rendered every species within the genus unpatentable). That is not the case here.

The PTO's focus on known methods for potentially isolating the claimed DNA molecules is also misplaced because the claims at issue define compounds, not methods. See *In re Bell*, 991 F.2d 781, 785, 26 USPQ2d 1529, 1532 (Fed.Cir.1993). In *Bell*, the PTO asserted a rejection based upon the combination of a primary reference disclosing a protein (and its complete amino acid sequence) with a secondary reference describing a general method of gene cloning. We reversed the rejection, holding in part that "[t]he PTO's focus on Bell's method is misplaced. Bell does not claim a method. Bell claims compositions, and the issue is the obviousness of the claimed compositions, not of the method by which they are made." *Id.*

[6][7][8] We today reaffirm the principle, stated in *Bell*, that the existence of a general method of isolating cDNA or DNA molecules is essentially irrelevant to the question whether the specific molecules themselves would have been obvious, in the absence of other prior art that suggests the claimed DNAs. A prior art disclosure of a process reciting a particular compound or obvious variant thereof as a product of the process is, of course, another matter, raising issues of anticipation under 35 U.S.C. § 102 as well as obviousness under § 103. Moreover, where there is prior art that suggests a claimed compound, the existence, or lack thereof, of an enabling process for making that compound is surely a factor in any patentability determination. See *In re Brown*, 329 F.2d 1006, 141 USPQ 245 (CCPA 1964) (reversing rejection for lack of an enabling method of making the claimed compound). There must, however, still be prior art that suggests the claimed compound in order for a *prima facie* case of obviousness to be made out; as we have already indicated, that prior art was lacking here with respect to claims 5 and 7. Thus, even if, as the examiner

stated, the existence of general cloning techniques, coupled with knowledge of a protein's structure, might have provided motivation to prepare a cDNA or made it obvious to prepare a cDNA, that does not necessarily make obvious a particular claimed cDNA. "Obvious to try" has long been held not to constitute obviousness. *In re O'Farrell*, 853 F.2d 894, 903, 7 USPQ2d 1673, 1680-81 (Fed.Cir.1988). A general incentive does not make obvious a particular result, nor does the existence of techniques by which those efforts can be carried out. Thus, Maniatis's teachings, even in combination with Bohlen, fail to suggest the claimed invention.

[9][10] The PTO argues that a compound may be defined by its process of preparation and therefore that a conceived process for making or isolating it provides a definition for it and can render it obvious. It cites *Amgen Inc. v. Chugai Pharmaceutical Co.*, 927 F.2d 1200, 18 USPQ2d 1016 (Fed.Cir.), cert. denied, 502 U.S. 856, 112 S.Ct. 169, 116 L.Ed.2d 132 (1991), for that proposition. We disagree. The fact that one can conceive a general process in advance for preparing an undefined compound does not mean that a claimed specific compound was precisely envisioned and therefore obvious. A substance may indeed be defined by its process of preparation. That occurs, however, when it has already been prepared by that process and one therefore knows that the result of that process is the stated compound. The process is part of the definition of the compound.*1560 But that is not possible in advance, especially when the hypothetical process is only a general one. Thus, a conceived method of preparing some undefined DNA does not define it with the precision necessary to render it obvious over the protein it encodes. We did not state otherwise in *Amgen*. See *Amgen*, 927 F.2d at 1206-09, 18 USPQ2d at 1021-23 (isolated/purified human gene held nonobvious; no conception of gene without envisioning its precise identity despite conception of general process of preparation).

We conclude that, because the applied references do not teach or suggest the claimed cDNA molecules, the final rejection of claims 5 and 7 must be reversed. See also *Bell*, 991 F.2d at 784-85, 26 USPQ2d at 1531-32 (human DNA sequences encoding IGF proteins nonobvious over asserted combination of references showing gene cloning method and complete amino acid sequences of IGFs).

[11] Claims 4 and 6 are of a different scope than claims 5 and 7. As is conceded by Deuel, they generically encompass all DNA sequences encoding

human and bovine HBGFs. Written in such a result-oriented form, claims 4 and 6 are thus tantamount to the general idea of all genes encoding the protein, all solutions to the problem. Such an idea might have been obvious from the *complete* amino acid sequence of the protein, coupled with knowledge of the genetic code, because this information may have enabled a person of ordinary skill in the art to envision the idea of, and, perhaps with the aid of a computer, even identify all members of the claimed genus. The Bohlen reference, however, only discloses a partial amino acid sequence, and thus it appears that, based on the above analysis, the claimed genus would not have been obvious over this prior art disclosure. We will therefore also reverse the final rejection of claims 4 and 6 because neither the Board nor the patent examiner articulated any separate reasons for holding these claims unpatentable apart from the grounds discussed above.

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One further matter requires comment. Because Deuel's patent application does not describe how to obtain any DNA except the disclosed cDNA molecules, claims 4 and 6 may be considered to be inadequately supported by the disclosure of the application. *See generally Amgen Inc. v. Chugai Pharmaceutical Co.*, 927 F.2d 1200, 1212-14, 18 USPQ2d 1016, 1026-28 (Fed.Cir.) (generic DNA sequence claims held invalid under 35 U.S.C. § 112, first paragraph), *cert. denied*, 502 U.S. 856, 112 S.Ct. 169, 116 L.Ed.2d 132 (1991); *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970) (Section 112 "requires that the scope of the claims must bear a reasonable correlation to the scope of enablement provided by the specification to persons of ordinary skill in the art."). As this issue is not before us, however, we will not address whether claims 4 and 6 satisfy the enablement requirement of § 112, first paragraph, but will leave to the PTO the question whether any further rejection is appropriate.

We have considered the PTO's remaining arguments and find them not persuasive.

CONCLUSION

The Board's decision affirming the final rejection of claims 4-7 is reversed.

REVERSED

C.A.Fed., 1995.

In re Deuel

51 F.3d 1552, 63 USLW 2624, 34 U.S.P.Q.2d 1210

10. RELATED PROCEEDINGS APPENDIX

None

CONCLUSION

Applicant respectfully submits that the various rejections of claims 16-21 and 24-30 under 35 U.S.C. §103(a) are improper and should be withdrawn. Fairness to Applicant requires reversal of the non-final rejection; therefore, such reversal is solicited.

Very respectfully,

SMITH & HOPEN, P.A.

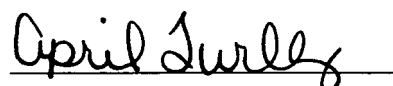
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